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Catabolismo muscular associado à quimioterapia: estudo dos mecanismos moleculares subjacentes

Chemotherapy-induced muscle wasting: exploring the underlying molecular mechanisms

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção de grau de Mestre em Bioquímica, ramo Bioquímica Clínica, realizada sob a orientação científica da Doutora Rita Maria Pinho Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e do Doutor Rui Gil da Costa, Investigador do Departamento de Engenharia Química da Faculdade de Engenharia da Universidade do Porto

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Dedico este trabalho à minha família por todo o apoio.

o júri

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palavras-chave Caquexia; cancro; cisplatina; catabolismo muscular.

resumo

A caquexia associada ao cancro (CC, do inglês: *cancer cachexia*) é uma síndrome metabólica que afeta mais do que 50% dos pacientes com cancro e, para a qual, atualmente, não existe tratamento. Uma das características principais desta síndrome é a perda involuntária e massiva de massa muscular, sendo esta associada a um mau prognóstico. A ativação de vias catabólicas e a presença de um estado inflamatório são responsáveis pelo processo de catabolismo. Assim, as intervenções terapêuticas direcionadas para a massa muscular ou para as vias ativadas pelo processo inflamatório serão, provavelmente, efetivas na diminuição dos efeitos devastadores da caquexia. Para além dos efeitos negativos do cancro na massa muscular, a quimioterapia tem vindo, também, a ser relacionada com o desenvolvimento e exacerbação desta síndrome. Infelizmente, os mecanismos responsáveis pelo catabolismo muscular induzido pela quimioterapia são pouco conhecidos e os estudos que têm sido realizados para a compreensão dos mesmos são escassos. Desta forma, na presente dissertação foi investigado, em animais saudáveis e com cancro da bexiga, o efeito do fármaco antitumoral cisplatina na massa muscular e, simultaneamente, foi estudado o potencial efeito do composto natural dimetilaminopartenolido (DMAPT) em contrariar o processo de catabolismo muscular. De um modo geral, a administração aguda de cisplatina (10 $mg.kg^{-1}$ no dia zero), em animais saudáveis, parece não induzir a síndrome caquexia. No entanto, o DMAPT parece originar hipertrofia no músculo através da modulação do processo inflamatório e do eixo TWEAK/NF-κB (do inglês: *tumour necrosis factor-related weak inducer of apoptosis*/*nuclear factor kappa light-chain-enhancer of activated B cells*). Relativamente aos animais com cancro, a cisplatina aparenta induzir o processo de catabolismo muscular e o DMAPT parece ser incapaz de reverter ou melhorar este processo, apesar da sua capacidade de reduzir a inflamação, que por sua vez, tem uma função importante no desenvolvimento da caquexia. Desta forma, é importante descobrir a interligação específica entre as vias ativadas pelo cancro e as que são induzidas pelos fármacos anticancerígenos, com o intuito de se desenvolverem estratégias que contrariem a elevada morbidade e mortalidade associadas a esta síndrome.

keywords Cachexia; cancer; cisplatin; muscle wasting.

abstract Cancer cachexia (CC) is a complex metabolic syndrome that affects more than 50% of cancer patients, and presently, no effective treatment exists. A key characteristic of this condition is the involuntary and massive loss of skeletal muscle mass, which is related with a poor prognosis. The activation of catabolic pathways and the presence of an inflammatory state are responsible for the wasting process. Therefore, therapeutic approaches directed to muscle or inflammatory pathways may probably be effective in decreasing the devastating effects of cachexia. Besides the negative effects of cancer itself on the skeletal muscle mass, chemotherapy has also been associated with the development and exacerbation of this process. Unfortunately, the mechanisms responsible for the muscle wasting process induced by chemotherapy are unclear and few studies have been devoted to this specific problem. In this way, we investigated the effect on the skeletal muscle mass of the widely-used chemotherapeutic agent cisplatin in healthy and urothelial carcinoma-induced animals, and simultaneously, studied the putative role of the natural compound dimethylaminoparthenolide (DMAPT) in counteract the muscle wasting process. In overall, an acute administration of cisplatin (10 $mg.kg^{-1}$ at day zero), in healthy animals, seems to be incapable of induce a cachectic syndrome. Nevertheless, DMAPT appears to induce muscle hypertrophy through the modulation of the inflammatory process and the tumour necrosis factor-related weak inducer of apoptosis/nuclear factor kappa light-chain-enhancer of activated B cells (TWEAK/NF-κB) axis. However, when cancer is involved, cisplatin can induce muscle wasting and DMAPT looks unable to revert or ameliorate this process, despite its capability to diminish the inflammation, which in turn has an important role in the development of cachexia. In this way, it is important to ascertain the specific relation between the pathways activated by cancer itself and the ones induced by the anti-cancer drugs, in order to develop strategies to counteract the high morbidity and mortality associated with this condition.

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ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E binding protein
Act A	Activin A
ActRIIB	Activin A type IIB receptor
ALK4	Activin A type IB receptor
ALK5	Activin A type IB receptor
ALP	Autophagy-lysosome pathway
AMPK	5´ adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
Atg	Autophagy-related genes
ATM	Ataxia telangiectasia mutated
ATP7A	Copper-transporting adenosine triphosphatase 1
ATP7B	Copper-transporting adenosine triphosphatase 2
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ATR	Ataxia telangiectasia mutated and RAD3-related protein
BBN	*N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine
BMI	Body mass index
Bnip3	B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3
BSA	Bovine serum albumin
CC	Cancer cachexia
CDDP	*cis*-diammine-dichloro-platinum
CHK1	Checkpoint kinase 1
CK2	Casein kinase 2

COX-2	Cyclooxygenase 2
CRP	C reactive protein
Ctr1	Copper transporter 1
DMAPT	Dimethylaminoparthenolide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
E1	Ubiquitin-activating enzymes
E2s	Ubiquitin-carrier or conjugating proteins
E3s	Ubiquitin-protein ligases
ECL	Enhanced chemiluminescence
eIF4E	Eukaryotic translation initiator
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinases
FDA	Food and Drug Administration
Fn14	Fibroblast growth factor-inducible 14
FoxO	Forkhead box O
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GDF	Growth differentiation factor
GH	Growth hormone
GHS	Growth hormone secretagogue
GHS-R1a	Growth hormone secretagogue receptor
gp130	Glycoprotein 130
GSH	Glutathione

IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
IκBs	Inhibitors of nuclear factor kappa light-chain-enhancer of activated B cells
IKK	Inhibitors of nuclear factor kappa light-chain-enhancer of activated B cells kinase
IL-1β	Interleukin 1 beta
IL-1R	Interleukin 1 receptor
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
I.P.	Intraperitoneally
IRS1	Insulin receptor substrate 1
JAK	Janus kinase
JNKs	c-JUN N-terminal kinases
LC3	Microtubule-associated protein light chain 3
LIF	Leukemia inhibitor factor
MAFbx	Muscle atrophy F-box protein
MAPK	Mitogen-activated protein kinase
MMP	Metalloproteinase
MMR	Mismatch repair
MST1	Mammalian sterile 20-like protein kinase 1
Mstn	Myostatin
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2

MuRF1	Muscle-specific RING-finger 1
NBD	Nemo-binding domain
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa light-chain-enhancer of activated B cells
Oct2	Organic cation transporter 2
p70S6K	p70 S6 kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
RAPTOR	Regulatory-associated protein of mammalian target of rapamycin
RICTOR	Rapamycin-insensitive companion of mammalian target of rapamycin
RNA	Ribonucleic acid
RPS6	Ribosomal protein 6
sActRIIB	Soluble activin A type IIB receptor
SAPK	Stress-activated protein kinases
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
SIRT6	Sirtuin 6
SMD	Skeletal muscle differentiation
STAT	Signal transducers and activators of transcription
TGF-β	Transforming growth factor beta
TLS	Translesion synthesis
TNF-α	Tumour necrosis factor alpha
TNF-αR	Tumour necrosis factor alpha receptor
TWEAK	Tumour necrosis factor-related weak inducer of apoptosis

UPP	Ubiquitin-proteasome pathway

I. INTRODUCTION

Cancer is the second leading cause of death worldwide and its occurrence is expected to increase because of the growth and aging of the population. [1,2] In 2012, about 14.1 million new cancer cases and 8.2 million deaths were estimated to have occurred. [2] In 2015, the number of deaths increased to over 8.7 million. [1] This complex disease is characterized by an uncontrolled growth of abnormal cells that have become capable to invade adjacent tissues and colonize other organs. [3] A common and debilitating complication of cancer is a metabolic syndrome termed cachexia. [4] The etiology of this syndrome is not well established and, therefore, no standardized assessment procedure exists nor therapies to prevent or treat cancer cachexia (CC). [5–7] This condition is characterized by the progressive wasting of fat tissue and skeletal muscle, despite adequate nutrition, thus negatively affecting the prognosis and quality of life of cancer patients. [4,8] Indeed, one of the main features of cachexia is the involuntary loss of skeletal muscle mass, known as muscle wasting, which contributes to the functional muscle impairment observed in cancer patients and to the difficulties related with the clinical delivery of chemotherapy. [9,10] Cancer treatments, such as chemotherapy, may also contribute to the development of cachexia. [11] In fact, chemotherapy negatively alters patient's nutritional state, and also, decreases the body weight. [12,13] Some cytotoxic drugs may generate their own cachexia-like side-effects, through the increased activity of various mediators involved in the molecular pathways underlying muscle wasting. [12–14] Nevertheless, few studies have been dedicated to the comprehension of the association between cancer treatments and the development of cachexia, which is responsible for more than 20% of all cancer-related deaths. [4,11,12,15] Therefore, the preservation of body weight through the prevention and treatment of skeletal muscle wasting may increase survival chances of cancer patients and improve their quality of life. [16]

1. THE IMPACT OF CHEMOTHERAPY IN CANCER TREATMENT

In the middle of the 20th century, chemotherapy emerged. [17] One of the first events that lead to the development and use of chemotherapeutic agents was performed by Sidney Farber, the father of the modern era of chemotherapy, in 1948. [18,19] He reported that folic acid antagonists could induce temporary remission in child patients with acute leukemia. [20] Since then, chemotherapy has become one of the principal treatment approaches in cancer treatment, influencing the treatment and survival of these patients. [17,21] In fact, chemotherapy is effective in the treatment of several malignancies, including choriocarcinoma, Hodgkin's and non-Hodgkin's lymphoma and germ cell, small cell lung, ovarian and testicular cancer. [17,22] This therapeutic approach usually involves the

administration of anticancer drugs in a standardized treatment regimen, which is specific for the type of cancer, with the aim of inducing tumour cell death. [13,23]

Chemotherapeutic drugs can be classified into alkylating agents (e.g. cisplatin and busulfan), antitumour antibiotics (e.g. mitoxantrone and bleomycin), antimetabolites (e.g. aminopterin and cytarabine), mitotic inhibitors (e.g. vinblastine and paclitaxel), topoisomerase inhibitors (e.g. etoposide and topotecan), corticosteroids, hormones and antagonists (e.g. tamoxifen and bicalutamide) and biologically targeted agents including tyrosine kinase inhibitors, antibiotics and other pathway inhibitors. [21] The agents that are frequently used induce cell death through apoptosis, mitotic catastrophe and premature senescence. The cytotoxic agents damage the deoxyribonucleic acid (DNA) or other critical molecules, which activate cellular effector systems, including the apoptotic machinery in cancer cells. However, cytotoxic chemotherapy is associated with many deleterious side-effects, such as myelosuppression and gastrointestinal and pulmonary toxicity. [15]

Conventional chemotherapy is frequently related with the development of drug resistance and systemic toxicity, which limits its therapeutic effectiveness. [24] This resistance to chemotherapeutics can be divided into intrinsic or acquired. [25] In intrinsic resistance, resistance-mediating factors pre-exist in the bulk of the tumour cells, making the treatment ineffective. Contrarily, in acquired resistance, the tumour is sensitive in the beginning of the treatment, but resistance is developed in the course of the therapy. This acquired resistance can be caused by mutations that appear during the treatment and by numerous other adaptive responses, including activation of alternative compensatory pathways and increased expression of the therapeutic target. Thus, alternative approaches are being development in order to decrease or abolish these therapy-related complications. [24] The improvements made so far include the use of chemotherapy in combination with other treatment modalities, such as immunotherapy, angiogenesis inhibition therapy, differentiation therapy and radiotherapy. These multiple treatment protocols have the aim to eliminate the complications related with cancer cell resistance to a specific drug or class of drugs and to decrease toxicity linked to high-dose chemotherapy. The drugs presently used for cancer treatment are not tumour-specific, and therefore, are frequently related with severe side-effects, such as immunosuppression and cardiotoxicity. Additionally, it is possible that chemotherapy may directly promote cachexia; however, the mechanisms involved are less well described. [11,15] Among the chemotherapeutic drugs related to cachexia, attention has been given to cisplatin; nevertheless, few studies have been addressed to the comprehension of the mechanisms responsible for cisplatin-induced muscle wasting. [13,15] Thus, this will be the agent studied in the present work.

1.1. The chemotherapeutic effect of cisplatin

Cisplatin (*cis*-diammine-dichloro-platinum^{II} or *cis*-[$PtCl_2(NH_3)_2$] or CDDP), an organic platinum-based chemotherapeutic agent, is one of the most potent and effective broad-spectrum anticancer drugs used. [13,23,26,27] It contains a platinum core with two amine nonleaving groups and two chloride leaving groups. [19] This anticancer agent was synthetized for the first time in 1845 and its structure deduced in 1893 by Alfred Werner. However, it was only in the 1960s that cisplatin was discovered as an anticancer drug and in 1978 was approved by the Food and Drug Administration (FDA) for the treatment of testicular and bladder cancer. [23,28] Nowadays, cisplatin integrates the standard of care for the treatment of several human tumours including head and neck, ovary and bladder cancer. [29] As a matter of fact, treatment with cisplatin has good efficacy in the therapy of these types of cancer, and also, of cervical cancer and can cure over 90% of all testicular cancer cases. [19] Consequently, these types of cancer received better prognosis, becoming less life threatening. [23] Cisplatin, which is administered intravenously, frequently results in an initial therapeutic success related to partial responses or disease stabilization. [28] Nevertheless, the use, efficacy and therapeutic profile of this anticancer agent are limited due to side-effects in normal tissues. [23,30] Such reversible and irreversible side-effects include nephrotoxicity, neurotoxicity, ototoxicity, hypomagnesemia, gastrointestinal toxicity, myelosuppression, hair loss, nausea and emesis. [15,27,30,31] The substantial neurotoxicity expresses as peripheral neuropathy, cachexia and anorexia. [32] In fact, it is usually observed in cancer patients treated with cisplatin a significant body weight loss, mainly due to muscle atrophy. [33] These complications frequently intensify the pain, anorexia and cachexia induced by the tumour itself. [32] Cisplatin-related toxicities are dose-dependent, so overdose may cause significant morbidity and/or mortality. [23] The incidence of cisplatin overdose is not known and, until now, general guidelines to manage a cisplatin overdose or a specific antidote do not exist. [23]

Cisplatin is a DNA-damaging agent, being generally accepted that genomic DNA is the primary target of this drug. [30] Since the discovery of the clinical benefits of cisplatin, studies have been made in order to understand how this chemotherapeutic agent works. [26,34–37] There are some gaps in the knowledge of the mechanisms underlying cisplatin-induced DNA damage. [38] In mammals, the uptake of cisplatin occurs through passive diffusion or involving the active copper transporter 1 (Ctr1) or the organic cation transporter 2 (Oct2) (**Figure 1**). [39,40] This drug can also be exported through copper-transporting adenosine triphosphatase (ATPase) 1 (ATP7A) and copper- transporting ATPase 2 (ATP7B), and can also be inactivated by reduced glutathione (GSH) or metallothionein. [40]

It is important to notice that the intracellular concentration of cisplatin determines the extent of the DNA lesions and, consequently, the efficacy of apoptosis machinery activation. To react with DNA, the neutral cisplatin needs to be activated through a series of spontaneous aquation reactions involving the successive replacement of *cis*-chloro ligands of the drug with water molecules. [26] Therefore, once inside the cell, cisplatin undergoes aquation to form $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$, a process that is facilitated by the low cellular concentrations of chloride ions (approximately 2-10 mM when compared with approximately 100 mM in the extracellular milieu). [28,31] Moreover, the aquated form is a positively charged molecule more reactive towards the cellular targets, in particular with endogenous nucleophiles, including GSH, methionine, metallothioneins, proteins (via their sulfhydryl groups on cysteines) and nitrogen donor atoms on nucleic acids. [28,30,38,39]

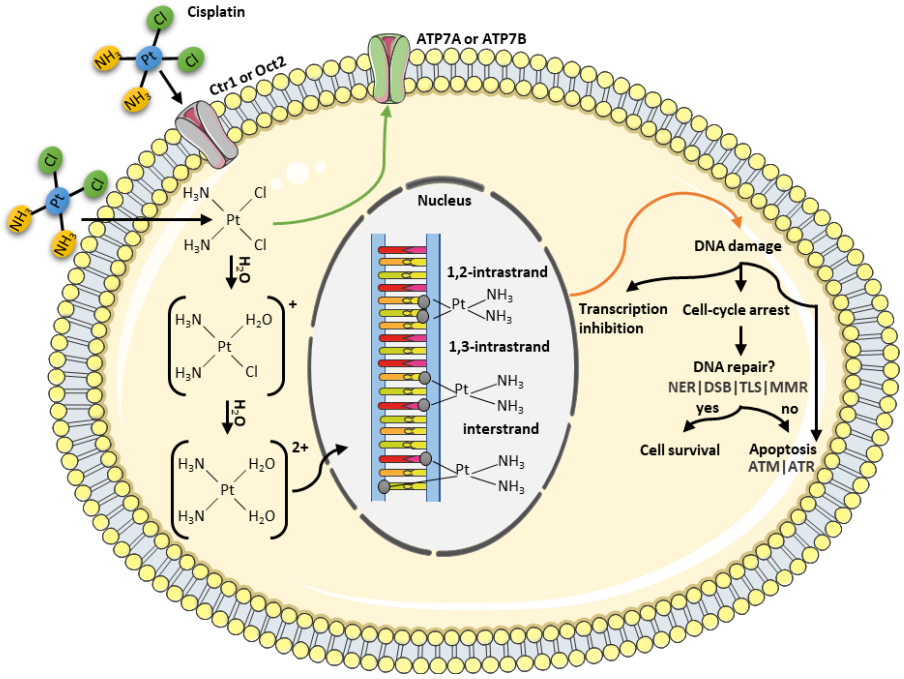

Figure 1. Mechanism of action of cisplatin. Cisplatin enters in the cell through passive diffusion or by the active copper transporter 1 (Ctr1) or the organic cation transporter 2 (Oct2). This drug can also be exported through the copper-transporting adenosine triphosphatase (ATPase) 1 (ATP7A) and the copper-transporting ATPase 2 (ATP7B). To react with deoxyribonucleic acid (DNA), the neutral cisplatin is activated by a series of spontaneous aquation reactions, forming $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$. Thus, the platinum binds to guanine and adenine residues, forming cisplatin-DNA adducts and inducing intrastrand and interstrand crosslinks. All of the cisplatin-DNA adducts distort the DNA structure and can be recognized by several repair pathways, including the nucleotide excision repair (NER) pathway, the double-strand break (DSB) repair, the translesion synthesis (TLS) and the proteins involved in the mismatch repair (MMR) system. If the degree of damage is limited, the several pathways activated culminate in DNA repair. Contrarily, if DNA repair fails or is overwhelmed by the extension of DNA lesions, cells undergo death (frequently apoptosis). In the process of apoptosis, two kinases that function as sensors of DNA damage are activated, named ataxia telangiectasia

mutated (ATM) and ATM and RAD3-related protein (ATR). Figure produced using the *Servier Medical Art*.

Due to the aquation of the chloride groups, the platinum binds to guanine and adenine (to a smaller extent) residues, forming cisplatin-DNA adducts and inducing intrastrand and interstrand crosslinks. [19,30,31] More specifically, the platinum atom of cisplatin forms covalent bonds with the N7 reactive centre on purine bases, resulting from this interaction between cisplatin and DNA: 1,2-intrastrand crosslinks involving adjacent guanine bases (GpG, 47-50%), 1,2-intrastrand crosslinks involving adjacent adenine and guanine bases (ApG, 23-28%), 1,3-intrastrand crosslinks involving nonadjacent guanines (*cis*-GNG), interstrand adducts (8-10%) and monofunctional binding to guanine (2-3%). [31,38,39] It has been indicated that the most important cisplatin-induced DNA lesions are the 1,2-intrastrand ApG and GpG, suggesting that these intrastrand adducts are responsible for most, if not all, cisplatin cytotoxicity. [26] All of the cisplatin-DNA adducts bend and unwind the DNA helix (i.e. distorting its structure), thus interfering with DNA replication and/or transcription, which result in DNA damage followed by cell-cycle arrest and cell death. [30,31,41] The Pt-DNA biofunctional adducts influence the cell life through two principal mechanisms – interaction with cellular proteins and inhibition of ribonucleic acid (RNA) transcription. [38] Among the various theories proposed to explain the mechanisms responsible for transcription inhibition, one of them is based on the blocking of the polymerase. This is due to the Pt-DNA crosslink, a barrier to translocation, whereby the bases in the platinated dinucleotide cannot rotate correctly in order to enter into the enzyme active site. [38]

The distortions in DNA can be recognized by numerous repair pathways. [28] It is thought that the nucleotide excision repair (NER) represents the principal mechanism for the removal of cisplatin adducts, since it is responsible for the repair of the intrastrand crosslinks. [28,40] With regard to the interstrand crosslinks, a complex mechanism for their repair is required, including the components of NER, double-strand break (DSB) repair and translesion synthesis (TLS). [40] Additionally, proteins involved in the mismatch repair (MMR) system also contribute to the recognition and resolution of cisplatin lesions. [28] If the degree of damage is limited, the cisplatin adducts activate several pathways that culminate in DNA repair. [26,28,40] One of these pathways involves the activation of cell cycle checkpoints, which temporally results in S-phase arrest, followed by inhibition of the cyclin A or B kinase, which in turn induces a durable G2/M arrest. [28] This process has cytoprotective effects, being re-established the DNA integrity through repair mechanisms and prevention of potentially abortive or abnormal mitoses. Contrarily, if DNA repair fails or

is overwhelmed by the extension of DNA lesions, cells undergo death (frequently apoptosis). [26,28,40]

When the cisplatin-induced DNA lesions lead to apoptosis, two kinases that function as sensors of DNA damage are activated, named ataxia telangiectasia mutated (ATM) and ATM and RAD3-related protein (ATR). [26,28] ATM is principally activated by cisplatin-induced DSBs, owing to continuous replication blockage, which causes collapse of replication forks, whereas ATR is activated due to stalled DNA replication forks. [40] Cisplatin preferentially activates ATR kinase, which phosphorylates tumour suppression protein p53 at serine-15 in order to initiate the activation of p53. [26] In addition, ATR activates the checkpoint kinase 1 (CHK1), which is the principal substrate and downstream effector of ATR. [26,28] CHK1 phosphorylates p53 on serine 20, stabilizing it. [28] Activated p53 signals to lethal functions through nuclear and cytoplasmic mechanisms that ultimately result in mitochondrial outer membrane permeabilization or increased signalling via death receptors followed by cell death. This process of apoptosis is initiated with the translocation of the cisplatin-induced Bax from the cytosol to the mitochondria. [26] In this organelle, a cascade of events occurs, including the release of apoptogenic factors (such as cytochrome c), which triggers the caspase-9/caspase-3 pathway, resulting in apoptosis. In addition, ATR has been linked to the activation of numerous specific branches of the mitogen-activated protein kinase (MAPK) cascade, including those mediated by extracellular signal-regulated kinases (ERK), c-JUN N-terminal kinases (JNKs or stress-activated protein kinases - SAPK) and p38 kinases, which phosphorylates p53 in various positions. [26,28] These MAPK members are involved in the regulation of cell proliferation, cell differentiation, cell survival and apoptosis. [26] The contribution of these pathways to the cytotoxic effects of cisplatin continues to be clarified. [28] Interestingly, ATM seems to be involved in cisplatin-induced cell cycle arrest but not cell death. Nonetheless, CHK2, which is the main downstream target of ATM, has been implicated in cisplatin-induced lethal signals in an ATM-independent manner. [28]

Cisplatin-based chemotherapy is related with high morbidity due to complications like malnutrition, cachexia, chemotherapy resistance and psychosocial distress, which adversely affects both quality of life and survival of cancer patients. [21,42,43] Cisplatin is regularly associated with marked side-effects, such as diarrhea, nausea, anorexia and fatigue. Cisplatin is a highly emetogenic chemotherapeutic agent, thus resulting in a decrease in appetite and, consequently, weight loss. [32] Regarding cachexia, cancer treatments may contribute to the development of this syndrome; however, its causative role is not completely understood, with few studies devoted to the comprehension of the

association between cancer treatment and the development of cachexia. [11,15] As a matter of fact, cancer patients with muscle wasting are more predisposed to develop severe drug-associated toxicity and present a poorer prognosis. [11] In opposition, patients with preserved muscle mass are normally more resistant to chemotherapy side-effects and may even tolerate higher doses of chemotherapy. [29] In fact, muscle fatigue is one of the most common side-effects of cisplatin during and after treatment. [13] Therefore, to increase the chances of a successful treatment and improve quality of life, the development of therapeutic strategies to counteract or attenuate these cancer-related complications is of enormous importance in the clinical practice. [13]

2. CANCER CACHEXIA: PATHOPHYSIOLOGIC MECHANISMS

A devastating complication of cancer is cachexia, a complex metabolic syndrome that means “bad condition”, from the Greek *kakos* (i.e. bad) and *hexis* (i.e. condition or appearance). [4,44] The common features among the several definitions of cachexia are weight loss, mostly because of the loss of skeletal muscle and body fat, and inflammation. [45] This life-threatening condition is related to numerous diseases, being very important in the setting of cancer since it affects more than 50% of all cancer patients and has a prevalence of about 86% in patients with advanced disease. [4,33,46] Cachexia is more prevalent in patients with solid tumours such as lung, pancreatic, colorectal or gastric cancer. [5,10] The development of cachexia is a strong predictor of morbidity and mortality, being responsible for 25-30% of all cancer-related deaths. Both weight loss and rate of weight loss correlate positively with mortality. [4] This wasting condition also decreases the efficacy and increases the toxicity of chemotherapy. [5,47] Presently, the moment when this condition appears in the clinical history of cancer patients is, generally, unpredictable. [48]

Pathophysiologically, CC is characterized by a negative protein balance (i.e. increased proteolysis and decreased protein synthesis) and energy imbalance (i.e. increased energy expenditure rate). [10,44] In fact, these profound alterations may be irreversible at the moment of evident weight loss. [48] Clinically, this syndrome is characterized by a weight loss of at least 5% in 6 months, in the presence of underlying illness and can be categorized as mild, moderate or severe corresponding to a weight loss of 5%, 10% or 15% of total body weight, respectively. [4] CC is associated with anorexia, early satiety, asthenia, fatigue, muscle weakness, skeletal muscle atrophy, low lean body mass, abnormal biochemistry (including increased inflammation, anaemia and low serum albumin) and impaired metabolic functions, like insulin resistance. [4,5,33,45,49]

Several mechanisms have been suggested to participate in the pathogenesis of CC, and therefore, it is considered a multifactorial and multi-organ syndrome, involving

metabolic pathways harboured in several organs (such as skeletal muscle and adipose tissue – both brown and white adipose tissues –, as well as brain, liver, gut and heart). [8,45] Although the etiology of this condition has not been established, several cellular and molecular mechanisms have been suggested, for instance, systemic inflammation, oxidative stress, metabolic disturbances and nutritional abnormalities. [7,8] By the fact that not all types of cancer promote CC, it is suggested that specific cancer cell-generated humoral factors are essential to the development of this wasting syndrome. [5] Despite the clinical studies performed so far and focused on the mechanisms of cachexia and targets for therapeutic interventions, no standardized assessment or treatments to prevent or treat cancer cachexia are currently available. [5,6,49] Thus, efforts continue to be made to better comprehend the molecular basis of CC envisioning the development of strategies to counteract the high morbidity and mortality associated with this syndrome. [50]

2.1. Cancer-induced muscle wasting

Skeletal muscle contributes to more than 40% of body weight – being the principal protein reservoir in the body – and seems to be the main wasted tissue in CC. [45,51] In fact, cachectic patients mainly demonstrate lean mass depletion particularly in the appendicular body compartment. [52] Since this area is predominantly composed of muscle mass, it seems that a specific loss of skeletal muscle occurs. Thereby, a key characteristic of cachexia is the involuntary loss of skeletal muscle mass, known as muscle wasting, which leads, along with other causes, to the functional impairment of skeletal muscle observed in CC and to the complications associated with the clinical delivery of chemotherapy. [9,10] Contrary to what happens in muscle atrophy caused by starvation or physical inactivity, muscle wasting in cachectic patients cannot be inverted by nutritional supplementation. [6] The loss of skeletal muscle mass seems to be closely related with the tumour size, stage and the type of anticancer drug used. [33] Several molecular mechanisms seem to be responsible for the cancer-induced loss of muscle mass, being modulated by the interaction between tumour and host factors. [5,33] Nevertheless, CC is not caused only by the cancer *per se*, being that the antineoplastic treatments, such as chemotherapy, may also contribute to the development and exacerbation of this syndrome. [53]

Cancer therapeutics are related with the development of systemic inflammation, intensification of the already-reduced energy and swallowing problems and anorexia due to nausea, which results in a negative effect on the nutritional intake of the patient. [12,53,54] Chemotherapy can induce anorexia, nausea, vomiting, mucositis, taste change or lethargy, thus interfering with the maintenance of the patient's nutritional state. [12] These symptoms are associated with the nature and course of the chemotherapeutic drugs used and some

cytotoxic drugs may generate their own cachexia-like side-effects. In fact, administration of the chemotherapeutic agents cisplatin or folfiri decreases body weight, including the skeletal muscle mass. [11,13] In addition, several studies reveal that cisplatin administration increases the activity of various mediators involved in the molecular pathways related to the muscle wasting process. [13,14] However, the association between CC and the effects of chemotherapy remains to be fully understood, being that different chemotherapy agents induce cachexia through different mechanisms, and so, future research is needed in order to understand their transduction pathways. [12,54]

Among the molecular pathways involved in the regulation of muscle mass are the ubiquitin-proteasome pathway (UPP), the autophagy-lysosome pathway (ALP), the insulin-like growth factor (IGF)-1/phosphatidylinositol-3-kinase (PI3K)/Akt (also known as PKB – protein kinase B)/mammalian target of rapamycin (mTOR) pathway, the myostatin pathway and the MAPK pathway (**Figure 2**). [5,10,13,29] The UPP seems to be responsible for the degradation of myofibrillar and specific regulatory proteins involved in muscle protein expression; the ALP targets the degradation of mitochondria and other cellular components; the overexpression of IGF-1/PI3K/Akt/mTOR pathway leads to muscle hypertrophy; and the activation of the myostatin pathway leads to atrophy. [5,13] Moreover, CC-related muscle wasting is accompanied by systemic inflammation. [5,13]

Systemic inflammation is an important host-related alteration that promotes muscle wasting, through the overrepresentation of pro-inflammatory cytokines and down-expression of anti-inflammatory cytokines. [52,55,56] In cancer, inflammation is a double-edged sword because, apart from the natural function of the immune system in regulating tumour growth, cancer cells induce the immune system to produce particular cytokines that stimulate tumour growth, progression and survival. [46] In fact, pro-inflammatory cytokines secreted both by immune cells or tumours can directly stimulate pathways that promote skeletal muscle protein turnover. [45] In addition, pro-inflammatory cytokines increase the metabolic rate due to the activation of thermogenesis, inhibit skeletal myocyte differentiation and decrease food intake. [48] Several pro-inflammatory mediators such as interleukin (IL) 1 beta (IL-1β), IL-6, interferon gamma (IFN-γ), transforming growth factor beta (TGF-β) and leukemia inhibitor factor (LIF) have been associated with the pathogenesis of muscle wasting, but emphasis has been given to the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α). [9] These molecules, principally TNF-α, are powerful activators of the prototypical nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) transcription factor (p65/p50). [55] In contrast with the upregulation of the pro-inflammatory cytokines, a decrease in the expression of anti-inflammatory cytokines simultaneously

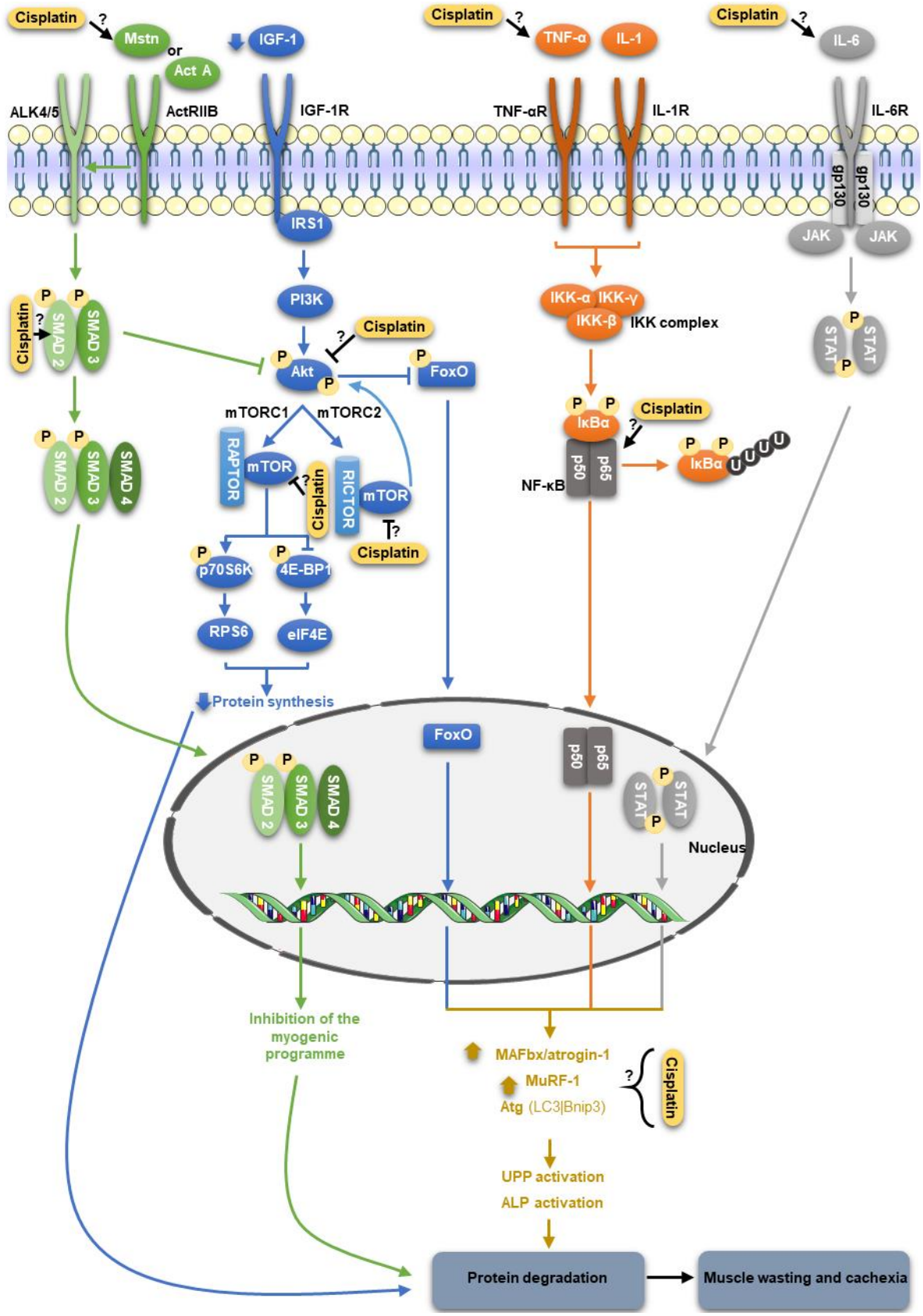

Figure 2. Signalling pathways modulated by cisplatin in skeletal muscle. In the myostatin (Mstn) pathway, this molecule binds to the activin A type IIB receptor (ActRIIB), resulting in its assembly with activin A type IB

receptor, ALK4 or ALK5. Consequently, SMAD2 and SMAD3 are activated by phosphorylation, which leads to the assembly with SMAD4. This heterodimer translocates to the nucleus and activate transcription of target genes. Thus, myostatin inhibits the myogenic programme, inducing muscle wasting. In addition, myostatin inhibits the insulin-like growth factor 1 (IGF-1)/phosphatidylinositol-3-kinase (PI3K)/Akt (also known as PKB – protein kinase B)/mammalian target of rapamycin (mTOR) pathway, and so, the levels of muscle atrophy F-box protein (MAFbx/atrogin-1), muscle-specific RING-finger 1 (MuRF1) and autophagy-related genes (Atg) increase, resulting in a decrease in protein synthesis and an increase in protein degradation. In the IGF-1/PI3K/Akt/mTOR pathway, IGF-1 activates the insulin receptor substrate 1 (IRS1) and then Akt blocks the repression of mTOR, which in turn, regulates the skeletal muscle mass by mTOR complex 1 and 2 (mTORC1 and mTORC2). In the mTORC1, which requires the regulatory-associated protein of mTOR (RAPTOR), occurs the phosphorylation and activation of p70 S6 kinase (p70S6K) and inhibition of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), which have as downstream targets the ribosomal protein 6 (RPS6) and the eukaryotic translation initiator (eIF4E), respectively, leading to protein synthesis. Regarding mTORC2, which binds to rapamycin insensitive companion of mTOR (RICTOR), this molecule phosphorylates Akt, resulting in maximum Akt activation. In addition, Akt phosphorylates and supresses the forkhead box O (FoxO) family of transcription factors. However, during catabolic diseases, this signalling pathway is silenced and, consequently, protein synthesis decreases and protein degradation increases. The prototypical nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) pathway can be activated by tumour necrosis factor alpha (TNF-α) and interleukin 1 (IL-1). These molecules lead to the activation of the IkB kinase (IKK) complex, which in turn, phosphorylates the NF-κB-bound inhibitors of NF-κB (IκBαs), which are target for polyubiquitination and degradation. The released NF-κB free dimers (p50/p65) translocate to the nucleus and induce the expression of MAFbx/atrogin-1 and MuRF1. Regarding interleukin 6 (IL-6), this cytokine can signalling through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, resulting in the suppression of protein synthesis. Legend: Act A: activin A | ALP: autophagy-lysosome pathway | Bnip3: B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3 | gp130: glycoprotein 130 | IGF-1R: IGF-1 receptor | IL-1R: IL-1 receptor | IL-6R: IL-6 receptor | LC3: microtubule-associated protein 1 light chain 3 | TNF-αR: TNF-α receptor | UPP: ubiquitin-proteasome pathway. Figure produced using the *Servier Medical Art*.

occurs, including IL-4, IL-10 and IL-12. [46] Furthermore, cisplatin treatment induces the expression of the pro-inflammatory cytokines TNF-α and IL-6 and diminishes the expression of the anti-inflammatory cytokine IL-10. [57] Despite the recognized importance of inflammation in the pathogenesis of CC, anti-inflammatory drugs had no success in the reversion of CC. [58] So, other biological processes might also be equally important in the pathogenesis of CC. [58]

2.1.1. Ubiquitin-proteasome pathway

The UPP is the principal pathway involved in the intracellular protein degradation of skeletal muscle fibres. [33] This pathway is composed by ubiquitin-activating enzymes (E1), ubiquitin-carrier or conjugating proteins (E2s) and ubiquitin-protein ligases (E3s), and involves two successive steps. [33,51] Firstly, occurs the polyubiquitination, that is, the covalent attachment of ubiquitin chains to the targeted protein substrate. This process

implies the sequential action of the E1, E2 and E3. [51] Subsequently, this complex can be recognized by the 26S proteasome, which digests the substrate into peptides. [33,51] Two muscle-specific E3 ligases – muscle-specific RING-finger 1 (MuRF1) and muscle atrophy F-box protein (MAFbx - also known as atrogin-1) – appear to be overexpressed in various catabolic conditions. The transcription factor forkhead box O (FoxO) is responsible for the increased transcription of MuRF-1 and MAFbx/atrogin-1. [59] These molecules are responsible for the ubiquitination and degradation of muscle proteins by the proteasome. [33] In fact, the deletion of MAFbx/atrogin-1 and MuRF1 protects the skeletal muscle in cases of experimental atrophy. [60] Contrarily, administration of cisplatin increases the expression levels of MAFbx/atrogin-1 and MuRF1. [13] The UPP is up-regulated by several cytokines, hormones (such as glucocorticoids) and myostatin, and inhibited by insulin or IGF-1. [60] It is thought that the elevated activity of this pathway in cachexia is induced by the activation of FoxO and NF-κB transcription factors, inducing the MuRF-1 and MAFbx/atrogin-1, and so, the proteasome activity. [61]

2.1.2. Autophagy-lysosome pathway

Autophagy may occur through macroautophagy, microautophagy, selective macroautophagy and microautophagy. [62] Macroautophagy (hereafter referred as autophagy) is used by the skeletal muscle to transport cytoplasm, organelles and proteins to the lysosome for degradation. [63] In this process occurs the sequestration of cytoplasmic components (e.g. long-lived or malfunctioning proteins and obsolete or damaged organelles) into double-membrane vesicles (i.e. autophagosomes), which subsequently fuse with lysosomes and their contents are digested by hydrolases and recycled. [62,64] The principal steps of this process – autophagy initiation, nucleation and lysosome fusion/degradation – are controlled by a family of evolutionary and conserved autophagy-related genes (Atg) proteins. [63] Autophagy occurs at physiological levels in all eukaryotic cells in order to remove misfolded proteins and damaged organelles and to prevent protein aggregates accumulation. However, the excessive and abnormal activation of this process during catabolic states exacerbates the process of muscle wasting, with the consequent impairment of myofiber homeostasis, leading to an excessive removal of cellular components needed for normal activities. [65,66] Indeed, abnormal autophagic protein degradation has been associated with several human diseases, including cancer. [67] Accordingly, components of the wasting process seem to mediate autophagy, including Akt, mTOR, pro-inflammatory cytokines and NF-κB activation. [62,63] In addition, FoxO3 has been implicated in the control of the autophagic degradation pathway in skeletal muscle, through the regulation of the expression of key Atg, including the microtubule-associated

protein 1 light chain 3 (LC3) and B cell lymphoma 2/adenovirus E1B 19-kDa protein-interacting protein 3 (Bnip3). [63]

The FoxO family is constituted by three members, being of notice FoxO1 and FoxO3 that are probably activated in all types of atrophy and can induce the expression of UPP and Atg, including LC3 and Bnip3, which are important markers of autophagosome formation. [12,16,68] Actually, activation of FoxO3 alone is sufficient to activate proteolysis through UPP and autophagy and, consequently, to cause significant atrophy. [16] Both FoxO1 and FoxO3 activity is strongly controlled by various post-translational modifications. On the one hand, FoxO3 is stimulated by phosphorylation by mammalian sterile 20-like protein kinase 1 (MST1) or 5´-adenosina monophosphate-activated protein kinase (AMPK). On the other hand, FoxO3 transcriptional activity is inhibited through phosphorylation by Akt, deacetylation by sirtuin 1 (SIRT1), ubiquitination or binding to JUNB or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). [16]

2.1.3. IGF-1/PI3K/Akt/mTOR pathway

IGF-1 signalling is possibly the best-characterized pathway related to muscle hypertrophy. [45] In most cells, this pathway controls cell division. [16] However, in the non-dividing muscle fibres, the IGF-1/PI3K/Akt/mTOR pathway promotes protein synthesis and inhibits protein degradation. Thus, IGF-1 activates insulin receptor substrate 1 (IRS1)-PI3K-Akt signalling, and then Akt blocks the repression of mTOR, which in turn, regulates skeletal muscle mass by two different complexes, termed mTOR complex 1 and 2 (mTORC1 and mTORC2). [45,69] In the mTORC1, which requires an adaptor protein named regulatory-associated protein of mTOR (RAPTOR), occurs the phosphorylation and activation of p70 S6 kinase (p70S6K) and inhibition of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), which have as downstream targets the ribosomal protein 6 (RPS6) and the eukaryotic translation initiator (eIF4E), respectively, leading to protein synthesis. [69–71] Regarding mTORC2, which binds to rapamycin-insensitive companion of mTOR (RICTOR), this complex phosphorylates Akt at serine 473 through a mechanism of positive feedback, thus allowing maximum Akt activation. [71] However, mTORC2 inhibition induces autophagy mainly through FoxO3. [69,72] Furthermore, Akt phosphorylates and supresses the FoxO family of transcription factors, preventing their translocation to the nucleus and the expression of the two ubiquitin ligases, MAFbx/atrogin-1 and MuRF1, and autophagy genes. [56] Nevertheless, during catabolic diseases, this signalling pathway is silenced, and consequently, protein synthesis decreases whereas FoxO-mediated proteolysis (through FoxO-mediated expression of the atrogene programme) and fibre atrophy increases. The inhibition of IGF-1/PI3K/Akt/mTOR pathway can occur due to increased levels of myostatin

or its homologue activin A. Additionally, administration of cisplatin also decreases phosphorylation of the proteins Akt and mTOR. [13,57]

2.1.4. Myostatin pathway

Myostatin (also known as growth differentiation factor (GDF) 8), an autocrine/paracrine cytokine that negatively regulates skeletal muscle mass and growth, is a TGF-β-family ligand that is predominantly expressed and released by skeletal muscle. [16,45,73,74] Cardiac muscle and adipose tissue exhibit low levels of this molecule. This ligand binds to the high-affinity activin A type IIB receptor (ActRIIB) on muscle membranes, resulting in its assembly with the activin A type IB receptor, ALK4 or ALK5. [16,73,75] Consequently, the two transcription factors SMAD2 and SMAD3 are activated by phosphorylation, which leads to the assembly of SMAD2/3 with SMAD4. [75] This heterodimer is able to translocate to the nucleus and activate transcription of target genes. Thus, myostatin inhibits the myogenic programme, the process responsible for the generation of myoblasts in order to originate skeletal muscle tissue, and so myoblast proliferation decreases. [45,56] This means that satellite cells, the stem cells responsible for muscle fibres proliferation and differentiation, are inhibited. [16] In addition, protein synthesis decreases, due to the inhibition of the IGF-1/PI3K/Akt/mTOR pathway by myostatin, and therefore, the mRNA levels of MAFbx/atrogin-1 and MuRF1 increase and degradation-related gene expression is activated. [44,45,56] This disinhibition of FoxO also leads to the upregulation of autophagy genes. [70] So, protein degradation increases and protein synthesis decreases. [45]

Though the physiological and pathophysiological mechanisms that regulate myostatin secretion in several conditions are mostly unknown, it is known that FoxO1, NF-κB, SMAD2, SMAD3 and SMAD4 can increase myostatin expression. [16] The released myostatin acts in an autocrine and paracrine manner, facilitating its catabolic effects. Other factors associated with TGF family members, such as activin A and GDF11, can stimulate SMAD2 and SMAD3 production through their binding to ActRIIB, thus acting like myostatin and limiting normal muscle growth and inducing muscle wasting. [16] Furthermore, administration of cisplatin increases the levels of phosphorylated SMAD2. [13]

2.1.5. NF-κB pathway

NF-κB is a transcription factor present in several cell types, such as in the mature skeletal muscle and, through the regulation of the expression of cyclin D1, inhibits vertebrate skeletal muscle differentiation (SMD). [76] Additionally, NF-κB is an activator of anti-apoptotic genes. [77] The mammalian NF-κB family is composed by five proteins –

RelA (p65), RelB, c-Rel, NF-κB1 (p50 and p105) and NF-κB2 (p52 and p100) - and has a crucial role in controlling innate and adaptive immunity. [78,79] These five proteins regulate the transcription of target genes by associating with each other, creating homo- and heterodimeric complexes, being that the p50 and p65 proteins form the principal prototypical heterodimer present in almost all the cell types. [78,80] Transactivation of the NF-κB can be started by several stimuli associated with various biological processes, for instance, inflammation. [55] In addition, activation of this pathway also accelerates inflammation. [12] Under physiological conditions, the activity of NF-κB is regulated; nevertheless, in cachexia this molecule is constitutively activated. [74]

In skeletal muscle, the potential targets of this factor are anti-apoptotic genes. [77] In non-stimulated cells, NF-κB proteins are present in the cytoplasm, forming a complex with inhibitor proteins collectively known as inhibitors of NF-κB (IκBs). [81] The majority of agents that activate NF-κB use a common pathway where, in a general way, occurs the phosphorylation of IκBs, followed by ubiquitination, and successively, degradation of IκBs by the proteasome. [79,81] Thus, cell stimulation leads to the activation of the IkB kinase (IKK) complex. [77] This complex is constituted by two catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, IKK-γ/NEMO. Activated IKK phosphorylates the two NH_2-terminal serines (serine 32 and serine 36) in NF-κB-bound IκBαs, which are targeted for polyubiquitination and degradation. [9,77,81] The released NF-κB free dimers (p50/p65) translocate to the nucleus and bind to their cognate DNA-binding sites in order to regulate the transcriptional activation of hundred target genes, including cytokines, chemokines, stress response proteins and numerous enzymes, such as those related to protein degradation by the UPP. [77,79–81] This classic NF-κB signalling pathway is only one of two major pathways that activate NF-κB transcription factors. [77] The second pathway or alternative pathway, includes the specific activation of p52:RelB heterodimers, and it is required for the generation of secondary lymphoid organs and for B-cell maturation and survival. [77] Thus, it is not directly involved in innate immunity and inflammation, but might be involved in mammary carcinogenesis. Additionally, NF-κB can be activated by another pathway (that has a small role in the physiological NF-κB activation) independent of IKK that might contribute to skin carcinogenesis, given that it is activated by ultraviolet radiation. This pathway is based on the activation of casein kinase 2 (CK2), where IκBα degradation is induced by the phosphorylation of carboxy-terminal sites. [77]

It has been demonstrated that NF-κB is a potent inducer of myostatin, but the specific mechanisms by which NF-κB promotes atrophy remain undefined. [16] Nonetheless, increased activity of NF-κB may lead to muscle wasting possibly through three

potential mechanisms: i) NF-κB may improve the expression of various proteins involved in the UPP, which in turn, are responsible for the degradation of specific muscle proteins; ii) NF-κB may increase the expression of inflammatory molecules that directly or indirectly promote muscle wasting; iii) NF-κB may affect the myogenic differentiation process that may be necessary for regeneration of atrophied skeletal muscles. [80] Thus, muscle-specific activation of NF-κB induced severe skeletal muscle atrophy similarly to the observed in CC. [9] Contrarily, selective blockade of NF-κB in muscle reduces the muscle wasting, and also, prolongs the survival in a mouse model (Lewis lung carcinoma) of CC. Additionally, NF-κB is involved in the deregulated activation and expansion of the satellite cell pool, which aggravates the wasting process. [46] This happens because satellite cells are incapable of complete differentiation. In addition, administration of cisplatin in healthy mice results in the activation of NF-κB (i.e. phosphorylation of its p65 subunit) and increases 4-fold the NF-κB DNA binding activity in myotubes. [14] Besides that, the upstream signals that control NF-κB function in muscle wasting are unknown. [16]

In cancer, as in other catabolic conditions, various pro-inflammatory cytokines such as TNF-α and IL-1 might induce the expression of NF-κB and, consequently, the upregulation of the expression of MURF1 and MAFbx/atrogin-1. [45] This pro-inflammatory response results particularly from the formation of p50/p65 dimers. [12] Recently, it was described an inflammatory cytokine named TNF-related weak inducer of apoptosis (TWEAK) that is strongly associated with muscle wasting. [16,54] This small pleiotropic cytokine is a member of the TNF-α superfamily and has several biological functions, such as induction of apoptosis and stimulation of pro-inflammatory cytokines. [54] This molecule causes NF-κB activation through binding to the surface fibroblast growth factor-inducible 14 (Fn14) receptor. [16] Moreover, TWEAK upregulates the expression of MAFbx/atrogin-1 and MuRF1 through the p38 MAPK and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Likewise, IL-6, which is secreted principally from tumour and immune cells, can signal through the JAK/STAT pathway, resulting in suppression of protein synthesis in muscle cells, since prolonged activation of this signalling is associated with muscle wasting observed in CC. [70,82] Until now, the efforts made to block a particular cytokine in order to prevent or slowdown the process of muscle wasting have been unsuccessful. [16,54] However, small-molecule inhibitors of IKK-β and suppressors of NF-κB activation have been described, but their specificity and potency are still uncertain. [16]

2.2. Therapies applied in the management of cancer cachexia

There is no approved treatment for cachexia. [10] Multimodal approaches consisting in nutritional, metabolic and pharmacological treatments (e.g. with corticosteroids and progestational drugs) have been used to prevent CC; however, these therapies have not demonstrated to be clinically effective. [33,83] Nutritional supplements alone do not reverse cachexia and resistance training, which may improve lean body mass and muscle strength, it is not usually prescribed for cancer patients. [29] Furthermore, pharmacological treatments as, for example, progestational agents, are commonly used; however, besides merely improving fat mass, can cause adrenal insufficiency, deep vein thrombosis and hypogonadism. [29] Likewise, there are no approved treatment for muscle wasting. [84] Nevertheless, in the field of CC management, the number of clinical trials and studies is increasing, with several agents under investigation (**Figure 3**), including the neutralization of crucial inflammation mediators (which have the benefit of influencing multiple steps that are also responsible for other characteristics of cachexia, such as anorexia, pain, fatigue and anemia), improvement of anabolic or inhibition of catabolic signals (which may improve the transformed energetic pathways, resulting in a more efficient function of muscle cells). [44,84,85]

Although inflammation is a leading cause of wasting, few randomized clinical trials have been directed in this field. [86] Clarithromycin and thalidomide have been suggested as potential CC treatments, due to their anti-inflammatory properties. Treatment with clarithromycin significantly decreases serum levels of IL-6, increases body weight and increases the survival of patients with non-small cell lung cancer. [87] Treatment with thalidomide attenuates weight loss due to a decrease in lean body mass loss in patients with pancreatic cancer. [88] However, the mechanism by which this agent works is not known, but it is possible that results from the downregulation of pro-inflammatory cytokines, including TNF-α. Another proposed mechanism is the inhibition of NF-κB. [89]

The use of celecoxib, an oral selective cyclooxygenase-2 (COX-2) inhibitor, demonstrated a significant decrease in serum levels of TNF-α and increase in lean body mass in a phase II study. [90] Another promising approach for the treatment of CC is the use of an ActRIIB decoy receptor (soluble (s)ActRIIB), which is able of weight loss reversal and animal survival prolongation. [91] Furthermore, the inhibition of the ActRIIB pathway prevents the activation of key components involved in the UPP, including MAFbx/atrogin-1 and MuRF1. ActRIIB antagonists block the SMAD2 activation and reduce the FoxO activity (i.e. increases FoxO phosphorylation), suggestive of IGF-1/PI3K/Akt/mTOR pathway activation. Moreover, myostatin neutralizing antibody can attenuate skeletal muscle mass

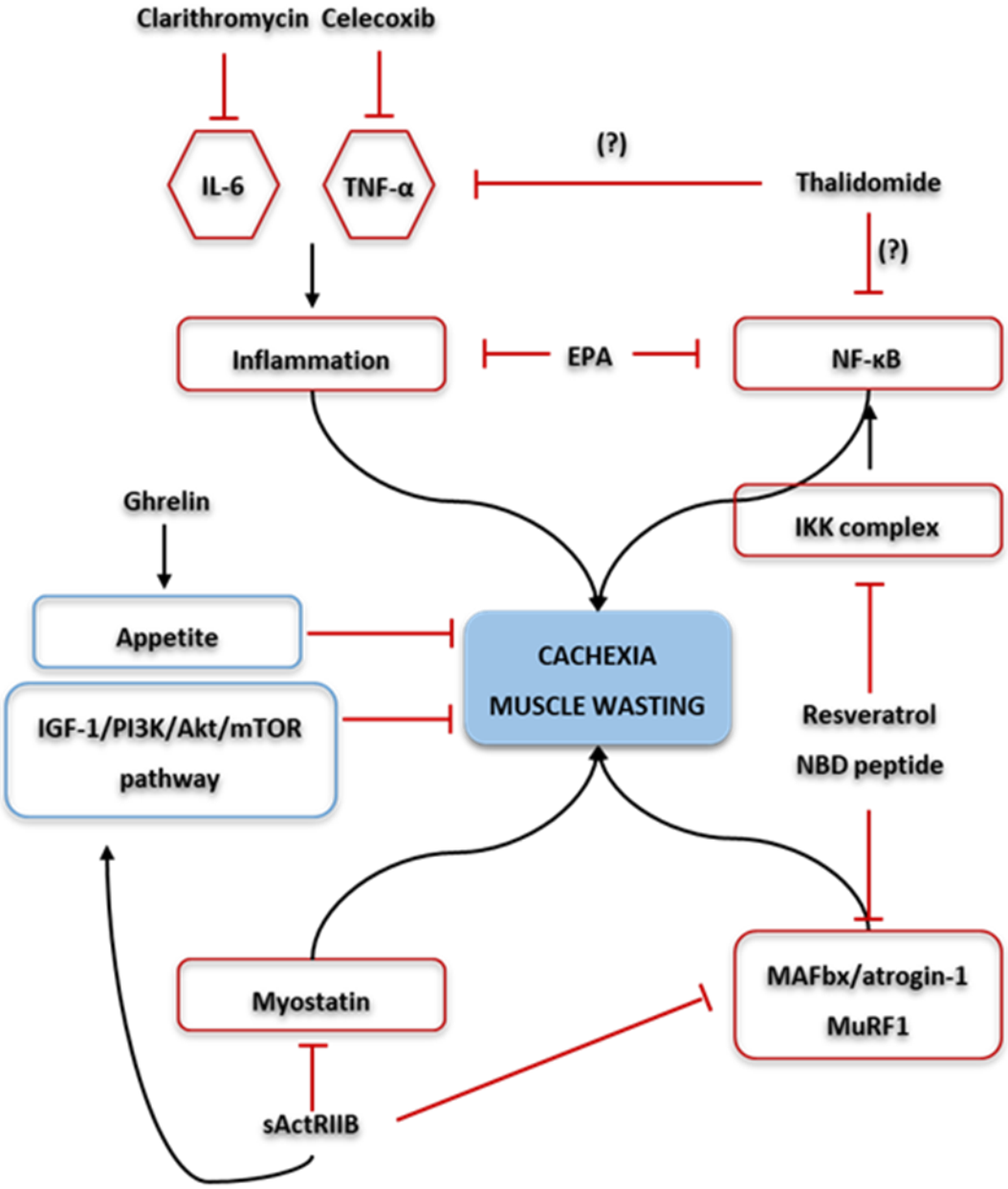

Figure 3. Possible therapeutic approaches for cachexia and muscle wasting. Ghrelin administration increases appetite and muscle mass. Clarithromycin decreases serum levels of interleukin 6 (IL-6), improving the cachectic status. Regarding thalidomide, this agent attenuates weight loss, possible (?) through downregulation of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and /or through the inhibition of prototypical nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB). In addition, the use of celecoxib demonstrated a significant decrease in serum levels of TNF-α and increase in lean body mass. The use of an activin A type IIB receptor (ActRIIB) decoy receptor (sActRIIB), results in the inhibition of the myostatin pathway, and also, prevents the activation of muscle atrophy F-box protein (MAFbx/atrogin-1) and muscle-specific RING-finger 1 (MuRF1). In addition, activate the insulin-like growth factor 1 (IGF-1)/phosphatidylinositol-3-kinase (PI3K)/Akt (also known as PKB – protein kinase B)/mammalian target of rapamycin (mTOR) pathway, thus preserving muscle mass. Resveratrol and the nemo-binding domain (NBD) peptide are inhibitors of the NF-κB pathway, and so, decrease the expression of MuRF1 and MAFbx/atrogin-1. Finally, the eicosapentaenoic acid (EPA), decreases pro-inflammatory cytokines release and inhibit NF-κB.

loss, both alone and in the context of chemotherapy. [92] In this perspective, restoration of the expression of sirtuin 6 (SIRT6) may be useful as a new therapeutic approach to diminish muscle wasting associated with chronic diseases since, under physiological condition, this molecule negatively regulates myostatin expression, possible through inhibition of the NF-κB signalling, promoting myogenesis. [74] Studies have been made in order to evaluate the use of agents that inhibit the nuclear translocation of NF-κB in the treatment of muscle wasting in CC. [93] In fact, resveratrol, an inhibitor of IKK, significantly decreases the NF-κB DNA-binding activity and significantly attenuates body weight loss and tumour growth rate. In addition, the administration of curcumin and rutin, which also inhibit NF-κB signalling, had a protective effect on muscle, improving survival and reducing inflammation [94]. Additionally, the treatment with the nemo-binding domain (NBD) peptide, a competitive inhibitor of the IKK complex, resulted in an increase in lean body and fat mass, through the inhibition of skeletal muscle NF-κB signalling and the decreased expression of MuRF1 and MAFbx/atrogin-1 in tumour-bearing mice, without evidences of toxicity to the liver or kidney. [95] Dimethylaminoparthenolide (DMAPT), which is the orally bioavailable analogue of parthenolide, a sesquiterpene lactone isolated from the medicinal herb feverfew, was also reported to inhibit the NF-κB activity and to present anti-inflammatory properties, and so, it seems to be a promising agent to counteract the wasting process. [96,97]

One of the most promising potential therapeutic option proposed for CC is an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R1a), named ghrelin. [10,29] Ghrelin, but also ghrelin receptor agonists, may improve muscle mass, strength, anorexia and weight loss in cancer patients, principally those receiving cisplatin-based chemotherapy. This 28-amino acid peptide, which contains a *n*-octanoyl modification on Ser^3 (i.e. biologically active), is produced, mainly, by the oxyntic cells of the stomach and it is a potent GH secretagogue (GHS). [32,83,98,99] Ghrelin administration increases appetite, fat and muscle mass, but mechanistically, the action of this hormone is not fully understood. [29] In other words, until now, it is not known if the antagonistic effect of ghrelin on weight loss induced by cisplatin is a consequence of the direct stimulatory effect of the hormone on hypothalamic neurons (where the GHS-R1a is present; more precisely on the arcuate nucleus) that regulate the appetite and/or of the indirect effect mediated by ghrelin's prokinetic properties (i.e. enhancement of gastrointestinal motility that may contribute to reduce the nausea and vomiting). [32,100] Nevertheless, it is thought that the effects produced by ghrelin administration are mediated by: i) an increase in food intake through the activation of the GHS-R1a in specific hypothalamic neurons; ii) a decrease in energy expenditure, also throughout hypothalamic effects, particularly by suppressing the

sympathetic nerve system output to brown adipose tissue, thus decreasing thermogenesis; iii) stimulation of GH release; iv) reduction of inflammation; v) direct effects in skeletal muscle and adipose tissue. [10,29,32,100] In fact, administration of ghrelin prevents the increased activation of the proteasome, as well as, the myostatin up-regulation. Furthermore, administration of ghrelin prevents the decrease in IGF-1 levels, being that both ghrelin and GHS promote the release of GH, resulting in an increase in the hepatic production of IGF-1. This increase in the levels of IGF-1 is related to an increase in protein synthesis and a decrease in proteolysis, thus possibly contributing to the preservation of muscle mass. [32,100] In addition, ghrelin prevents the increase in the levels of phosphorylated p38 and pro-inflammatory cytokines (e.g. TNF-α, IL-6 and IL-1β). [29,32] As a matter of fact, these cytokines and ghrelin have antagonistic effects on appetite and body weight. [98,100] Cytokines may directly decrease ghrelin production through the activation of anorexigenic pathways that lead to a reduction in food intake. In the other hand, ghrelin may supress TNF-α and IL-6 production, via the central inhibition of the inflammatory process. In addition, the administration of ghrelin stabilizes weight loss during treatment with cisplatin and induces a rapid weight gain after it. [32] Interestingly, in cancer-induced cachexia, active ghrelin levels are elevated and the same occurs after cisplatin administration, which suggests that resistance to the orexigenic effects of the endogenous ghrelin might occur. [32,98] However, this resistance should be partial, since further elevation in ghrelin levels in 3- to 4- fold from the baseline, seems to increase appetite and food intake. [98] Thus, various synthetic GHS have been developed and are presently in study for the treatment of CC. [100] Similarly, the synthetic and orally active orexigenic progestational agents megestrol acetate and medroxyprogesterone acetate have also been shown to improve food intake, promoting weight gain [12,44]. In addition, they decrease pro-inflammatory cytokines levels, including TNF-α, IL-1 and IL-6 [12]. The mechanism of action of these drugs is unknown and, unfortunately, they exhibit several limitations [44,86]. For example, the weight gain obtained is habitually composed of fat, and besides that, these drugs have a significant glucocorticoid character, promoting muscle wasting. Moreover, these agents are expensive, contraindicated in patients with hormone-sensitive diseases and have side-effects, including impotence in males [86].

The nutritional approach through the integration of formulated nutrient mixtures in the diet not only provides energy and protein but also counteracts CC-related inflammation. [53] Oral supplementation with eicosapentaenoic acid (EPA), an ω-3 polyunsaturated fatty acid present in fish oil, significantly decreases the weight loss rate in patients with advanced pancreatic cancer. [101,102] It is suggested that this effect is due to a decrease in pro-

inflammatory cytokine release and NF-κB inhibition. Likewise, docosahexaenoic acid has been studied as a therapeutic approach to CC. [53] In addition, branched chain amino acids (e.g. leucine, isoleucine and valine) have been shown to increase protein synthesis and decrease protein degradation in skeletal muscle, thus having a protective effect on muscle mass. [103]

Exercise training has been suggested to integrate the multimodal treatment approach to CC. [53] In fact, exercise may modulate skeletal muscle metabolism and inflammation. Treadmill exercise was reported to attenuate cisplatin-induced up-regulation of MuRF1 and MAFbx/atrogin-1 in a mouse model. [104] Voluntary wheel running also prevented lean body mass loss in cisplatin-treated mice, by abolishing the cisplatin-induced expression of MuRF-1, MAFbx/atrogin-1 and IL-6, as well as the repression of Akt and mTOR. [57] However, exercise training interventions are normally difficult to implement in the set of cancer since there are several factors that might limit the exercise capacity (e.g. anemia and chronic fatigue). [104]

Finally, the potential therapeutic approaches against muscle wasting should: i) modulate the expression or activity of circulating factors that induce the UPP and/or the NF-κB (e.g. TNF-α or IL-6), that inhibit muscle growth (e.g. myostatin) or that promote muscle hypertrophy (e.g. insulin or IGF-1); ii) target intracellular effectors involved in muscle wasting, including the NF-κB pathway or the transcription factor FoxO; and directly inhibit the UPP. [85] However, a limitation of the efforts to reduce muscle wasting is that the targeted molecules are not muscle-specific, and so, modification of their activity or expression may be related to side-effects that might deteriorate the clinical status of the patient. Thus, a possible strategy that could help to improve treatment is a combination of drugs with synergistic effects, allowing the use of sub-therapeutic doses of each drug and so decrease the possibility of injurious side-effects. Another strategy is the development of compounds that have as targets specific key factors involved in muscle wasting and that have no general functions outside the skeletal muscle. For instance, the E3s specifically induced during muscle wasting, such as MuRF-1, are potential options. Accordingly, the disruption of the interaction between the E3s and its substrate(s) may be the key to obtain specific and efficient inhibitors of the E3s. However, information such as the nature of the relevant substrates of the E3s and the structure of the E3-substrate pair to be targeted are not currently available. [33]

II. AIMS

The general aim of the present dissertation was to study the effect of the widely-used chemotherapeutic agent cisplatin on the skeletal muscle remodelling, and simultaneously, the putative therapeutic role of the natural compound DMAPT, since it can inhibit the NF-κB pathway and has anti-inflammatory properties. To accomplish this aim, two studies were made. In **study 1**, it was evaluated the effect, in healthy animals, of an acute administration of cisplatin on inflammatory mediators and signalling pathways that regulate the skeletal muscle mass and the influence of DMAPT on that effect. Concerning the **study 2,** it was ascertained, in the urothelial-carcinoma context, the effect of chronic administration of cisplatin alone and in combination with DMAPT on serum markers of catabolism.

III. MATERIALS AND METHODS

1. CHEMICALS

Cisplatin was purchased from TEVA Pharma (Porto Salvo, Portugal), DMAPT was kindly provided by Professor Christopher Sweeney (Dana-Farber Cancer Institute, Boston, EUA) and *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) was acquired from Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents and chemicals used were of highest grade of purity commercially available. Rabbit polyclonal anti-NF-κB p65 (ab16502), rabbit monoclonal anti-NF-κB p105/50 (ab32360), rabbit polyclonal anti-MuRF-1 (ab77577), rabbit polyclonal anti-myostatin (ab98337), mouse monoclonal anti-adenosine triphosphate (ATP) synthase (ab14730), rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab9485), rabbit polyclonal anti-IL-6 (ab6672), rabbit polyclonal anti-C reactive protein (CRP) (ab65842), rabbit polyclonal anti-ghrelin (ab64325), rabbit polyclonal anti-TWEAK (ab37170), rabbit polyclonal anti-metalloproteinase (MMP) 2 (ab37150) and rabbit polyclonal anti-MMP9 (ab38898) were acquired from Abcam® (Cambridge, UK). Rabbit polyclonal anti-atrogin-1 (AP2041) was acquired from ECM Biosciences (Versailles, Kentucky) and rabbit monoclonal anti-phospho-IκBα (#2859S) from Cell Signaling Technology (MA, USA). Anti-rabbit and anti-mouse horseradish peroxidase secondary antibodies (NA934V and NA931V, respectively) were purchased from GE Healthcare (UK).

2. EXPERIMENTAL PROCEDURES

In order to accomplish the proposed aims for this dissertation, the experimental methodology illustrated in **Figure 4** was followed. This research was divided in two studies: in **study 1** we evaluated the skeletal muscle remodelling induced by an acute administration cisplatin and related with the pro-inflammatory and catabolic cytokines profile in serum and the role of DMAPT on these effects; in **study 2** focus was given to serum alterations induced by chronic administration of cisplatin in cancer animals and also the effect of DMAPT on these alterations. All methods are clarified in the following sub-sections.

2.1. Animals

In **study 1**, six weeks-old female CD1-mice were divided in three experimental groups with five animals each: control, cisplatin and cisplatin plus DMAPT. The animals treated with cisplatin were intraperitoneally (i.p.) injected with 10 $mg.kg^{-1}$ of the drug at day zero, whereas 100 $mg.kg^{-1}$ of DMAPT was administered orally and daily, starting at day -1, in the respective animals. Mice were sacrificed after two weeks. Regarding **study 2**, an immune-competent mouse model of muscle-invasive (T2) bladder cancer induced by the tobacco-related agent BBN was used. Six weeks-old female CD-1 mice were utilized. The animals were divided in five experimental groups: control (n=15), BBN (n=19), BBN and

cisplatin-treated (n=18), BBN and DMAPT-treated (n=17) and BBN and the combination of cisplatin plus DMAPT (n=19). In the groups treated with cisplatin the animals were administered i.p. with 4.0 mg.kg^{-1} weekly, while the mice treated with DMAPT received 100.0 mg.kg^{-1} daily, for six weeks. All the procedures followed the 2010/63/EU animal welfare directive. In the day before the sacrifice, forelimb grip strength test was performed to evaluate muscle force using a grip strength meter, as previously reported. [105]

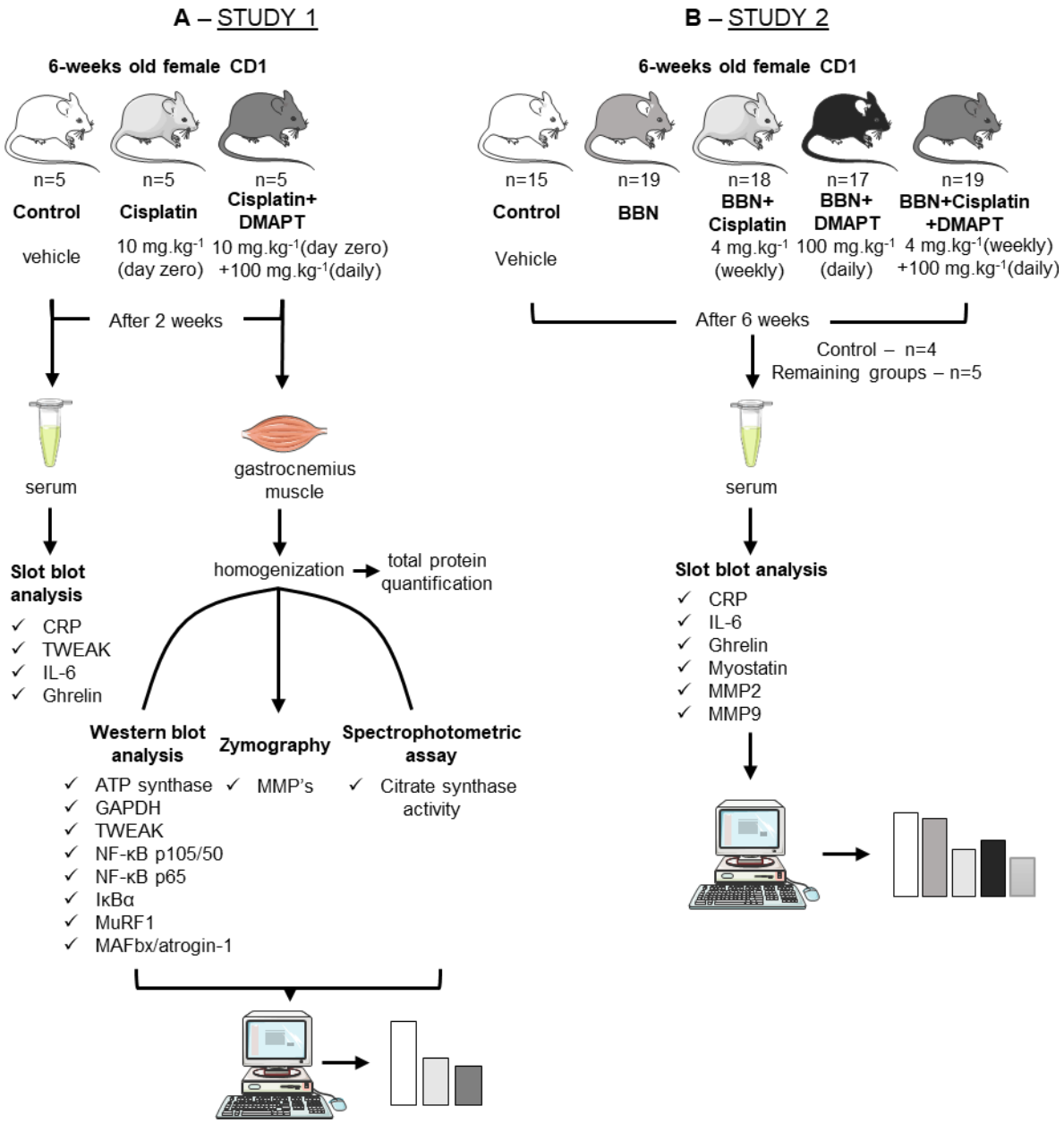

Figure 4. Schematic figure representing the experimental procedures performed in the (A) study 1 and (B) study 2. In study 1 the animals were divided in three groups – control (n=5), cisplatin (n=5) and cisplatin + dimethylaminoparthenolide (DMAPT) (n=5) –, whereas the study 2 was constituted by five experimental groups – control (n=15), N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) (n=19), BBN + cisplatin (n=18), BBN + DMAPT (n=17), BBN + cisplatin + DMAPT (n=19). In both studies serum analysis was done through slot blot assays,

while western blot analysis, zymography and spectrophotometric assay were only conducted in study 1 in order to analyse the gastrocnemius muscle. The proteins analysed are below the corresponding assays. Legend: ATP: adenosine triphosphate | CRP: C reactive protein | GAPDH: glyceraldehyde 3-phosphate dehydrogenase | IκBα: inhibitors of NF-κB | IL-6: interleukin-6 | MAFbx/atrogin-1: muscle atrophy F-box protein | MMP: metalloproteinase | MuRF1: muscle-specific RING-finger 1 | NF-κB: prototypical nuclear factor kappa light-chain-enhancer of activated B cells | TWEAK: tumour necrosis factor-related weak inducer of apoptosis. Figure produced using the *Servier Medical Art*.

2.2. Gastrocnemius muscle preparation and analysis

A portion of approximately 50 mg of gastrocnemius muscle was homogenized in 1 mL of 100 mM phosphate buffer (50 mM KH_2PO_4, 0.5% Triton X-100 and 200 mM PMSF), using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer. The obtained samples were preserved at -80ºC for biochemical analysis.

2.2.1. Total protein quantification

The protein content in the gastrocnemius muscle samples was assayed with the commercial kit *DC™ Protein Assay* (Bio-Rad®, CA, USA), according to the manufacture's recommendations and using bovine serum albumin (BSA) as a standard. Briefly, to 5 µL of gastrocnemius muscle homogenate or BSA standards was added 25 µL of reagent A' (prepared by the addition of 1 mL of reagent A and 20 µL of reagent S) and 200 µL of reagent B. After 15 minutes at room temperature, the absorbance was measured at 750 nm in a microplate reader (Multiskan GO, Thermo Fischer Scientific®, Northumberland, UK).

2.2.2. Spectrophotometric assay of citrate synthase activity

The citrate synthase activity was determined according to Coore and collaborators [106]. Firstly, the reaction mixture composed by 200 mM Tris pH 8.0, 10 mM acetyl-CoA, 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 0.1% (v/v) Triton X-100 was prepared. Secondly, to 5 µL of each sample was added 190 µL of the reaction mixture and the absorbance was read at 412 nm for approximately 2 minutes at 30ºC. Thirdly, 4 µL of 10 mM oxaloacetate was added to each well of the microplate and the absorbance was again read at 412 nm for approximately 2 minutes at 30ºC. Fourthly, the absorbance values of the second phase of readings for each well were plotted against time, being the slope of the equation (ΔAbsorbance412/min) divided by the molar absorption coefficient, at 412 nm, of the mercaptide ion formed (13.6 $mM^{-1}.cm^{-1}$). Finally, the acquired values were divided by the total protein content of each sample, obtaining the citrate synthase activity.

2.2.3. Western blot analysis

Certain volumes of gastrocnemius muscle samples equivalent to 50 μg of protein were diluted 1:1 (v/v) in reduction buffer (0.5 M Tris-HCl pH 6.8, 4% (w/v) SDS, 15% (v/v) glycerol, 0.04% (w/v) bromophenol blue and 20% (v/v) β-mercaptoethanol) and incubated at 100 ºC for 5 minutes, followed by electrophoresis on a 12.5% sodium dodecyl sulphate--polyacrylamide gel electrophoresis (SDS-PAGE) (prepared according to Laemmli [107]) during 45 minutes at 180 V in running buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS). Then, the gels were blotted onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) during 2 hours at 200 mA. After this process, protein loading was controlled by Ponceau S staining. Nonspecific binding was blocked in 5% (w/v) nonfat dry milk in TBS-T (100 mM Tris pH 8.0, 1.5 M NaCl and 0.5% Tween 20) during 1 hour at room temperature with agitation. Then, the membranes were incubated with the corresponding primary antibody [anti-NF-κB p65, anti-NF-κB p105/50, anti-MuRF-1, anti-atrogin-1, anti-TWEAK, anti-GAPDH, anti-ATP synthase and anti-phospho IκBα (diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T)] for 2 hours at room temperature and with agitation. After this, the membranes were washed 3 times (10 minutes each) with TBS-T, followed by incubation with anti-rabbit or anti-mouse peroxidase secondary antibody (diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T) for 2 hours at room temperature with agitation. Then, the membranes were again washed 3 times with TBS-T (10 minutes each). Lastly, immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (WesternBright™ ECL, advansta, CA, USA) according to the manufacturer's procedure. Images were recorded using X-ray films (Amersham™ Hyperfilm™ ECL, GE Healthcare), which were scanned using Gel Doc XR system (Bio-Rad®, CA, USA) and analysed with Image Lab software (Bio-Rad®, CA, USA, version 6.0.0,). The optical densities obtained were expressed in arbitrary units.

2.2.4. Gelatine zymography

Certain volumes of gastrocnemius muscle samples equivalent to 50 μg of protein were incubated on charging buffer (100 mM Tris pH 6.8, 5% SDS, 20% glycerol and 0.1% bromophenol blue) for 10 minutes at room temperature, in a proportion of 1:1 (v/v), followed by electrophoresis. In this procedure, a 10% SDS-PAGE separation gel with 0.1% of gelatin was used and the gels were run during 1 hour at 125V. Then, the gels were incubated in renaturation buffer (2.5% Triton X-100) for 1 hour at room temperature and with agitation, followed by incubation in development buffer (50 mM Tris pH 8.8, 5 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.4, 0.02% (v/v) Triton X-100) for 30 minutes at room temperature and with

agitation. Immediately after this, the gels were again incubated in a new development buffer, but now overnight and at 37ºC. The zymography gels were then stained with a solution of Coomasie Brilliant Blue G250 (0.4% (w/v) Coomassie Brilliant Blue, 50% (v/v) ethanol and 10% (v/v) acetic acid), during 3 hours at room temperature and with agitation. Lastly, the gels were destained with 25% (v/v) ethanol and 5% (v/v) acetic acid with two changes of the solution. The gels were scanned using Gel Doc XR system (Bio-Rad®, CA, USA) and analysed with Image Lab software (Bio-Rad®, CA, USA, version 6.0.0). The optical densities obtained were expressed in arbitrary units.

2.3. Serum sample analysis

In order to analyse biochemical parameters in serum samples, 10 µL of each sample was diluted in 490 µL TBS 1x (10 mM Tris, pH 8.0, 0.15 M NaCl). Then, 50 µL of each of these samples were blotted onto a nitrocellulose membrane (Amersham™, Protan®, GE Healthcare), followed by Ponceau S staining for protein loading control. Nonspecific binding was blocked with 5% (w/v) nonfat dry milk in TBS-T, during 1 hour at room temperature with agitation. The membranes were incubated with the corresponding primary antibody [anti-IL-6, anti-TWEAK, anti-ghrelin, anti-CRP, anti-myostatin, anti-MMP2 and anti-MMP9 (diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T)] for 1 hour at room temperature and with agitation. After this time, the membranes were washed 3 times (10 minutes each) with TBS-T and incubated with anti-rabbit peroxidase secondary antibody (diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T) for 1 hour at room temperature with agitation. Membranes were then washed 3 times with TBS-T (10 minutes each). Immunoreactive bands were detected with ECL reagents (WesternBright™ ECL, advansta, CA, USA) according to the manufacturer's procedure. Images were recorded using X-ray films (Amersham™ Hyperfilm™ ECL, GE Healthcare), which were scanned using Gel Doc XR system (Bio-Rad®, CA, USA) and analysed with Image Lab software (Bio-Rad®, CA, USA, version 6.0.0,). The optical densities obtained were expressed in arbitrary units.

2.4. Statistical analysis

Values are presented as mean ± standard deviation for each experimental group. The statistical significance of the differences between the experimental groups was determined using a one-way analysis of variance (ANOVA) followed by the Turkey multiple comparisons post hoc test. Results were considered significantly different when $p<0.05$. These statistical analyses were performed with the GraphPad Prism® software for windows (version 6.01).

IV. RESULTS

1. THE EFFECT OF CISPLATIN OR CISPLATIN PLUS DMAPT ON CATABOLIC PHENOTYPE

In **study 1** were evaluated anthropometric, serum and skeletal muscle alterations induced by cisplatin or cisplatin plus DMAPT administration on healthy mice. The results of these analyses are described in the following sub-sections.

1.1. Characterization of animals' response to cisplatin or cisplatin plus DMAPT

Description of the animals used in this study concerning body weight, gastrocnemius muscle weight, spleen weight, kidney weight and gastrocnemius-to-body weight, spleen-to-body weight and kidney-to-body weight ratios is reported in **Table 1**. Administration of cisplatin plus DMAPT caused a significant reduction of body weight, when compared with the animals that received only cisplatin ($p<0.01$). Nevertheless, no significant differences were observed between groups on the gastrocnemius muscle weight; however, cisplatin and DMAPT exposure caused a significant increase of the ratio gastrocnemius-to-body weight when compared to the control ($p<0.05$) and cisplatin ($p<0.01$) groups, suggestive of muscle hypertrophy. Concerning the spleen, the animals treated with the combination of cisplatin and DMAPT had a significant decrease in its weight, when compared with the control ($p<0.01$) and cisplatin ($p<0.05$) groups; nonetheless, when the ratio between the spleen and the body weight is analysed, it is observed a significant decrease on the cisplatin plus DMAPT group only when compared with the control one ($p<0.01$). In addition, administration of cisplatin plus DMAPT reduced the kidney weight, comparing to the control and cisplatin groups ($p<0.01$) and the same occurred to the kidney-to-body weight ratio ($p<0.01$ vs. control and $p<0.05$ vs. cisplatin).

Table 1. The effect of cisplatin or cisplatin plus DMAPT on body weight, gastrocnemius muscle weight, spleen weight and kidney weight and on the ratios gastrocnemius-to-body weight, spleen-to-body weight and kidney-to-body weight. Values are expressed as mean ± standard deviation.

	Experimental groups		
	Control	**Cisplatin**	**Cisplatin + DMAPT**
Body weight (g)	30.51 ± 2.19	32.92 ± 2.32 **	27.22 ± 2.05
Gastrocnemius muscle weight (g)	0.256 ± 0.008	0.267 ± 0.034	0.260 ± 0.019
Gastrocnemius-to-body weight ($mg.g^{-1}$)	8.43 ± 0.50 *	8.08 ± 0.62 **	9.57 ± 0.57
Spleen weight (g)	0.195 ± 0.054 **	0.156 ± 0.022 *	0.090 ± 0.026
Spleen-to-body weight ($mg.g^{-1}$)	6.35 ± 1.66 **	4.72 ± 0.48	3.27 ± 0.74
Kidney weight (g)	0.580 ± 0.088 **	0.588 ± 0.055 **	0.404 ± 0.074
Kidney-to-body weight ($mg.g^{-1}$)	19.0 ± 1.7 **	17.8 ± 0.9 *	14.7 ± 1.7

** $p < 0.01$ vs. cisplatin + DMAPT and * $p < 0.05$ vs. cisplatin + DMAPT

The results of serum analysis are depicted in **Figure 5**. Cisplatin plus DMAPT administration caused a reduction in CRP levels, comparing with the cisplatin group ($p<0.05$, Figure 5. A). Likewise, cisplatin plus DMAPT administration caused a significant reduction of TWEAK serum levels, but when compared to the control group ($p<0.05$, Figure 5. B). Regarding IL-6 (Figure 5. C) or ghrelin (Figure 5. D) circulating levels no significant differences were observed between groups.

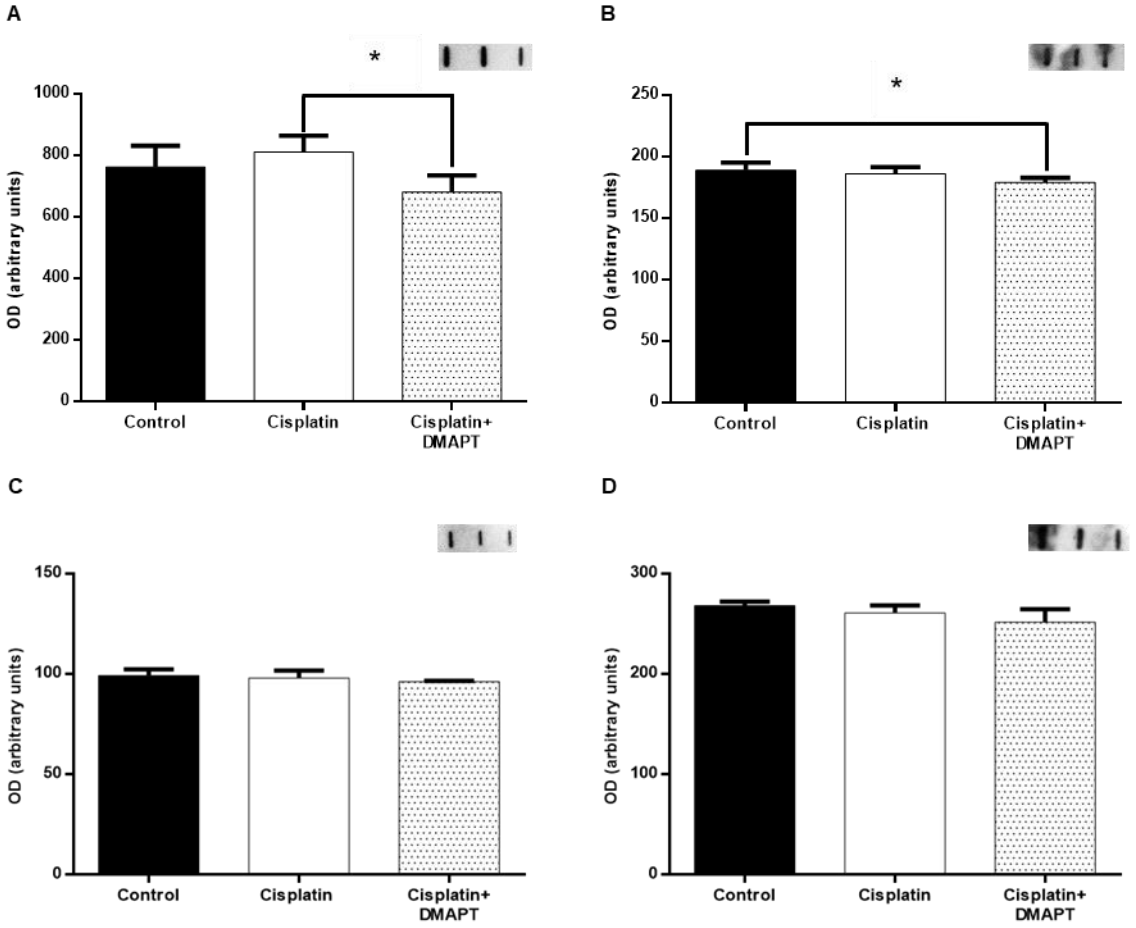

Figure 5. Effect of cisplatin or cisplatin plus DMAPT administration on (A) CRP, (B) TWEAK, (C) IL-6 and (D) ghrelin serum levels. Representative immunoblots are presented above the corresponding graphs. Values are expressed as mean ± standard deviation. (* $p<0.05$)

1.2. Analysis of the gastrocnemius muscle response to cisplatin or cisplatin plus DMAPT administration

The metabolic profile of gastrocnemius from each of the experimental groups in study is represented in **Figure 6**. No significant differences were observed between groups on ATP synthase (Figure 6. A) or GAPDH (Figure 6. B) levels, nor ATP synthase/GAPDH ratio (Figure 6. C). Likewise, no differences were observed in the activity of citrate synthase (Figure 6. D). So, the therapies studied seem to have no impact on the metabolic phenotype of gastrocnemius.

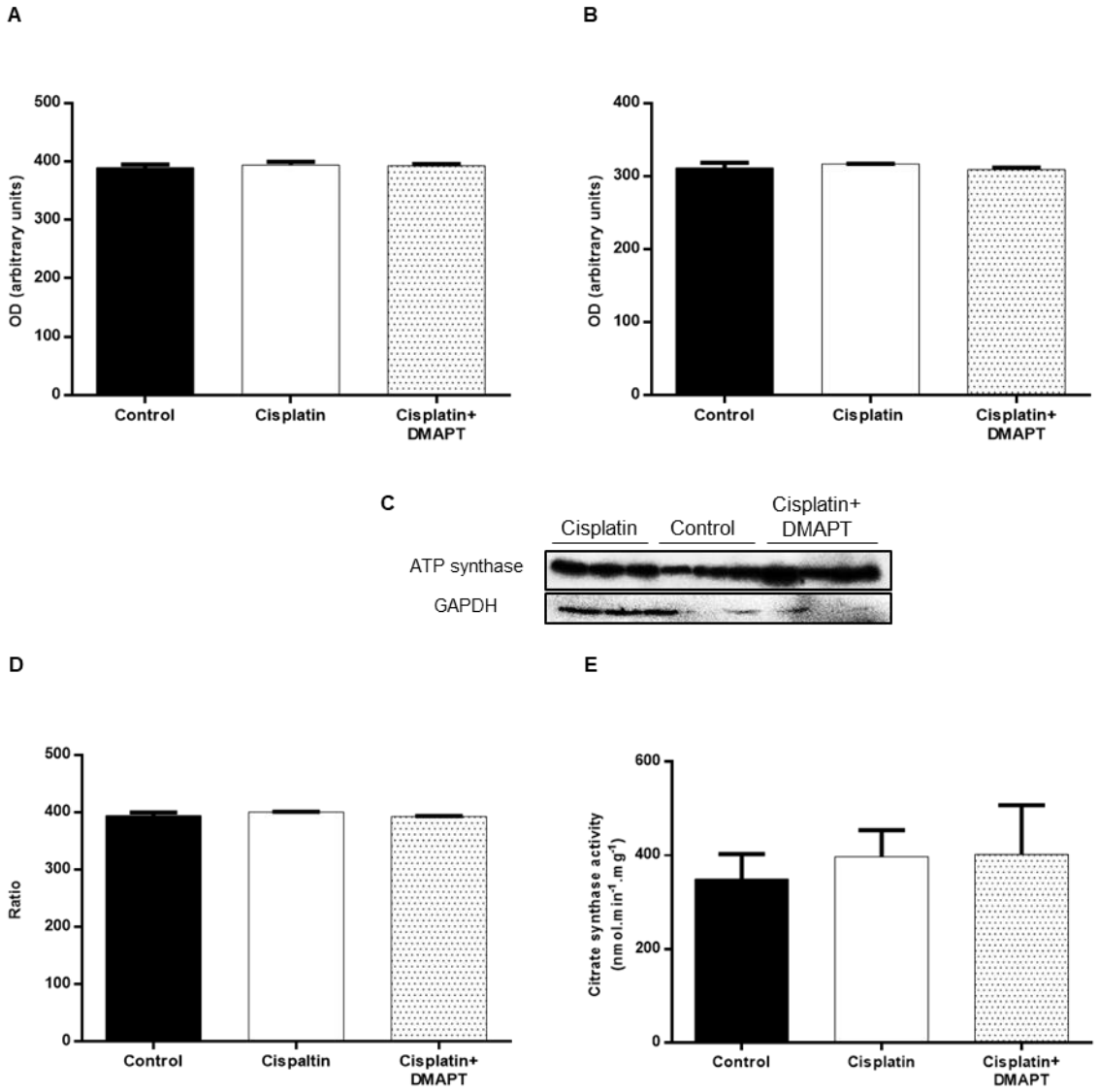

Figure 6. Effect of cisplatin or cisplatin plus DMAPT administration on the muscle expressions of (A) ATP synthase and (B) GAPDH. Representative immunoblots are presented in (C). It is also presented the (D) ATP synthase/GAPDH ratio. In (E) the citrate synthase activity can be observed. The values are expressed as mean ± standard deviation.

The TWEAK/NF-κB axis was also studied, considering the expected effect of DMAPT on NF-κB signalling, [96,108] and the results are depicted in **Figure 7**. Regarding TWEAK (Figure 7. A), it was observed in the animals exposed to the combination of cisplatin and DMAPT a significant increase in its expression, when compared to the cisplatin group ($p<0.05$). Administration of cisplatin plus DMAPT also caused a significant increase in the expression of the subunit p105/50 of NF-κB, relatively to the cisplatin group ($p<0.05$, Figure 7. B). However, no differences were observed in the levels of the subunit p65 of NF-κB (Figure 7. C) or in the levels of the inhibitor IκBα (Figure 7. D). So, DMAPT seems to induce the formation of p50 homodimers in the gastrocnemius muscle, possibly through the TWEAK-induced activation of Fn14.

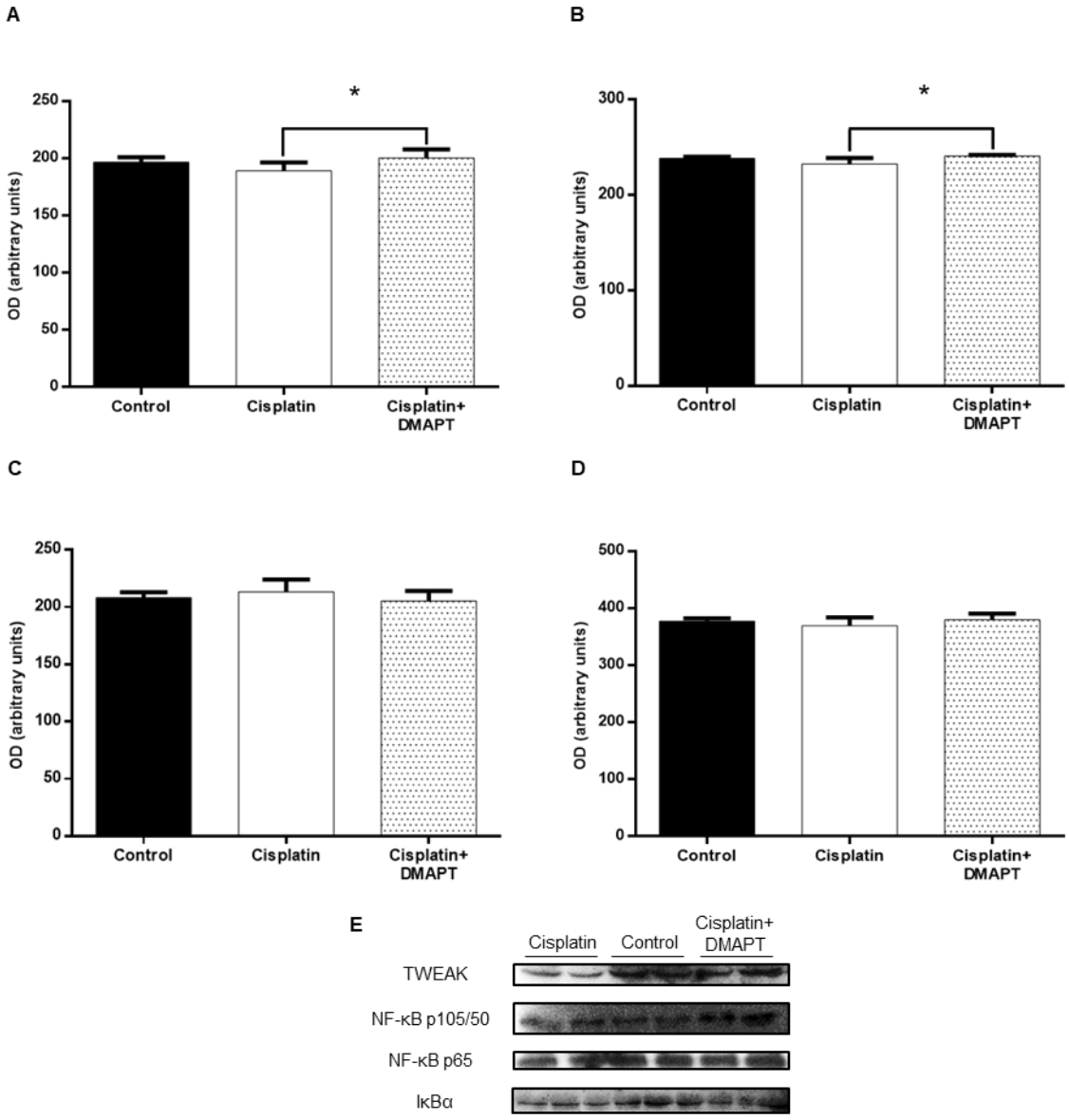

Figure 7. Effect of cisplatin or cisplatin plus DMAPT administration on the muscle expressions of (A) TWEAK, (B) NF-κB p105/50, (C) NF-κB p65 and (D) IκBα. Representative immunoblots are presented in (E). The values are expressed as mean ± standard deviation. (* $p<0.05$)

Concerning the proteolytic activity, the results obtained can be observed in **Figure 8**. No significant differences were observed between the experimental groups in the levels of MuRF1 (Figure 8. A) or MAFbx/atrogin-1 (Figure 8. B). Nevertheless, the analysis of the zymography gel (Figure 8. C) evidenced a significant reduction of the activity of the band with 72 kDa (which is predicted to be MMP2 according to Uniprot) in cisplatin plus DMAPT group, comparing with the cisplatin one ($p<0.05$, Figure 8. D). It was also analysed the myostatin expression by western blotting to evaluate its putative contribution to muscle catabolism, but no signal was detected in the blots.

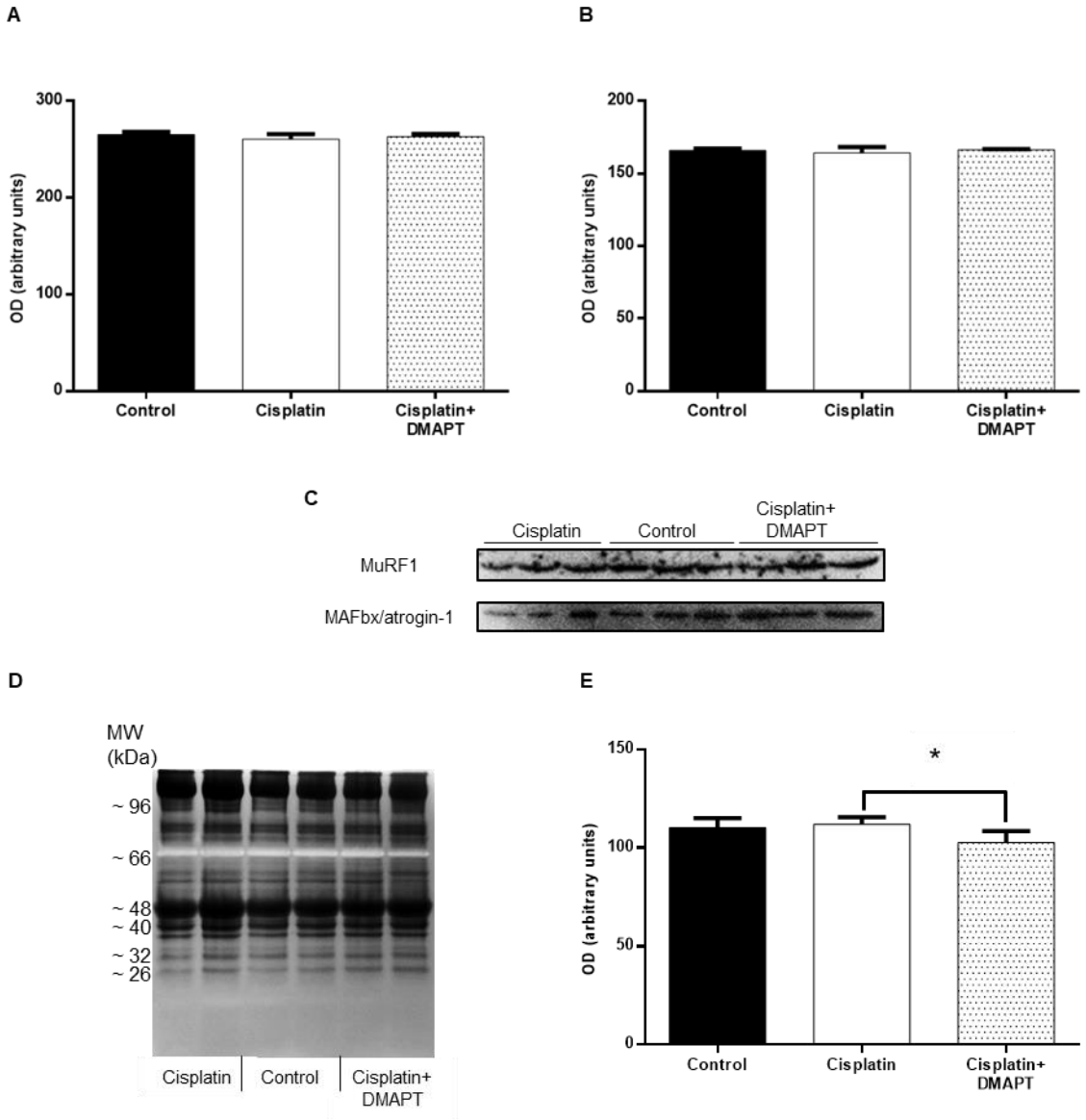

Figure 8. Effect of cisplatin or cisplatin plus DMAPT administration on the muscle expression of (A) MuRF1 and (B) MAFbx/atrogin-1. Representative immunoblots are presented in (C). (D) Representative zymography gel, evidencing one band with proteolytic activity. In (E) is presented the semi-quantitative analysis of the proteolytic activity for each group. The values are expressed as mean ± standard deviation. (* $p<0.05$)

2. THE EFFECT OF CISPLATIN, DMAPT OR CISPLATIN PLUS DMAPT ON BLADDER CANCER-INDUCED ANIMALS

In **study 2,** the effect of cisplatin, DMAPT or the combination of these two agents in animals' anthropometric parameters and serum profile was explored. During the protocol, one DMAPT-treated and one cisplatin-treated mouse died.

2.1. Characterization of animals' response to cisplatin, DMAPT or cisplatin plus DMAPT administration

In **Table 2** is reported the description of the animals used in this study concerning body weight, gastrocnemius muscle weight, spleen weight and gastrocnemius-to-body weight and spleen-to-body weight ratios. In cancer-induced animals exposed to cisplatin or cisplatin plus DMAPT occurred a reduction on body weight, comparatively to BBN animals ($p<0.0001$) and to the control ones ($p<0.0001$ vs. BBN + Cisplatin and $p<0.001$ vs. BBN + Cisplatin + DMAPT). Contrarily, in BBN animals administered with DMAPT occurred an increase of body weight, comparing with BBN plus cisplatin ($p<0.0001$) or BBN plus cisplatin plus DMAPT ($p<0.001$) animals. Regarding gastrocnemius muscle, in both BBN plus cisplatin animals or BBN plus cisplatin plus DMAPT animals a significant decrease in this muscle weight occurred, comparing to the control ones ($p<0.05$). Nevertheless, no significant differences were observed in the gastrocnemius-to-body weight ratio. Regarding the spleen, when comparing the BBN plus cisplatin group with the control ($p<0.01$) or BBN groups ($p<0.0001$) it is observed a significant weight decrease. Likewise, in BBN plus cisplatin plus DMAPT animals a decrease on spleen weight was observed, comparing with the control ($p<0.05$) or BBN ($p<0.01$) or BBN plus DMAPT ($p<0.01$) ones. Concerning spleen-to-body weight ratio, a significant decrease was observed in BBN plus cisplatin-treated animals comparing with the BBN plus DMAPT littermates ($p<0.05$).

Table 2. The effect of cisplatin or DMAPT or the combination of cisplatin plus DMAPT on body weight, gastrocnemius muscle weight, spleen weight and the ratios gastrocnemius-to-body weight and spleen-to-body weight. Values are expressed as mean ± standard deviation.

	Experimental groups				
	Control	**BBN**	**BBN + Cisplatin**	**BBN + DMAPT**	**BBN + Cisplatin + DMAPT**
Body weight (g)	33.9 ± 3.2 [a,c]	34.8 ± 3.5	27.3 ± 3.6 [b]	33.8 ± 2.5 [a,c]	28.6 ± 4.5 [b]
Gastrocnemius muscle weight (g)	0.207 ± 0.037	0.188 ± 0.048	0.152 ± 0.046 [f]	0.193 ± 0.046	0.157 ± 0.051 [f]
Gastrocnemius-to-body weight ($mg.g^{-1}$)	5.18 ± 0.83	5.38 ± 1.25	5.23 ± 1.46	5.07 ± 1.32	5.08 ± 1.83
Spleen (g)	0.127 ± 0.031 [d]	0.137 ± 0.026 [e,a]	0.085 ± 0.035	0.138 ± 0.036 [e,a]	0.096 ± 0.030 [f]
Spleen-to-body weight ($mg.g^{-1}$)	3.77 ± 1.03	3.95 ± 0.76	3.14 ± 1.22 [g]	4.08 ± 1.04	3.32 ± 0.72

[a] $p<0.0001$ vs. BBN + Cisplatin; [b] $p<0.0001$ vs. BBN; [c] $p<0.001$ vs. BBN + Cisplatin + DMAPT; [d] $p<0.01$ vs. BBN + Cisplatin; [e] $p<0.01$ vs. BBN + Cisplatin + DMAPT; [f] $p<0.05$ vs. control; [g] $p<0.05$ vs. BBN + DMAPT

In **Table 3** is depicted the percentage of lesions observed in the animals (bladder tissue examination was performed by veterinarian pathologists). BBN-induced animals or BBN plus cisplatin or BBN plus DMAPT treated ones showed invasive and muscle invasive lesions, whereas in the control or BBN plus cisplatin plus DMAPT groups no lesions were observed.

Table 3. Characterization of the experimental groups used in study 2 concerning the percentage of invasive and muscle invasive lesions.

	Experimental groups				
	Control	**BBN**	**BBN + Cisplatin**	**BBN + DMAPT**	**BBN + Cisplatin + DMAPT**
Invasive lesions (%)	0.00	42.1	11.1	35.3	0.00
Muscle invasive lesions (%)	0.00	15.8	5.6	17.6	0.00

It was also performed a grip strength test and the results are presented in **Figure 9**. No significant differences were observed between groups concerning the grip strength (Figure 9. A) or the grip strength-to-body weight ratio (Figure 9. B), suggesting that the conditions in study did not induced muscle fatigue.

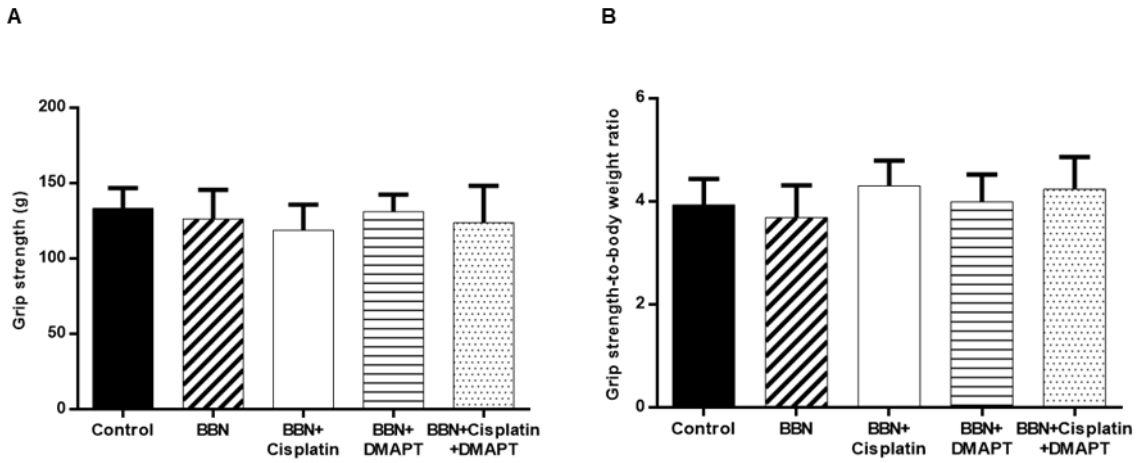

Figure 9. Effect of cisplatin or DMAPT or cisplatin plus DMAPT administration on the (A) grip strength or (B) grip strength-to-body weight ratio of BBN animals. The values are expressed as mean ± standard deviation.

The results of serum analysis are depicted in **Figure 10**. No significant differences were observed in CRP (Figure 10. A), ghrelin (Figure 10. C), myostatin (Figure 10. D), MMP2 (Figure 10. E) or MMP9 (Figure 10. F) circulating levels. Regarding IL-6 content, a significant increase occurred in the BBN-treated animals that received cisplatin, when compared to the control ones ($p<0.05$, Figure 10. B).

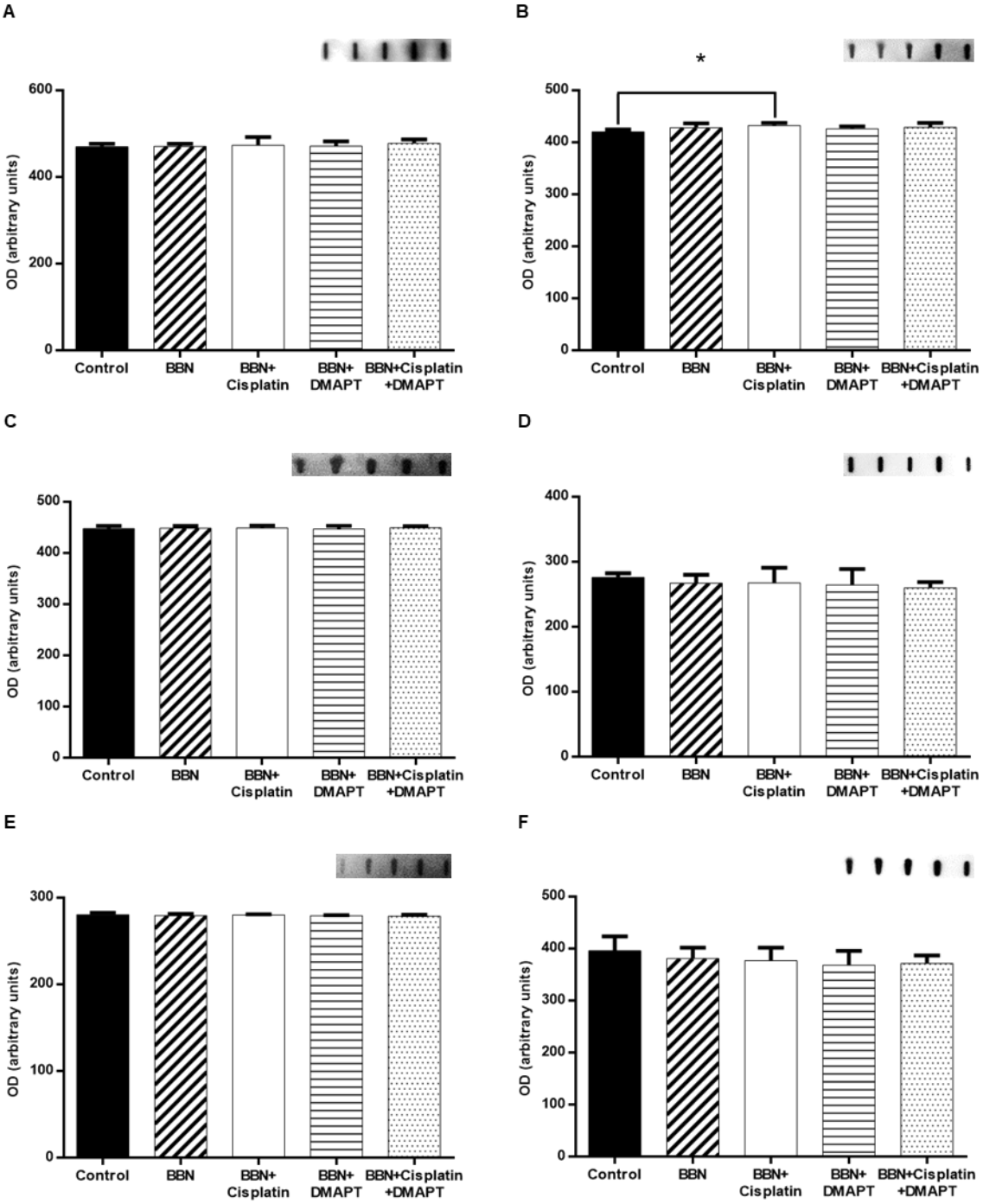

Figure 10. Effect of cisplatin or DMAPT or cisplatin plus DMAPT administration on the serum levels of (A) CRP, (B) IL-6, (C) ghrelin, (D) myostatin, (E) MMP2 or (F) MMP9. Representative immunoblots are presented above the corresponding graphs. The values are expressed as mean ± standard deviation. (* $p<0.05$)

V. DISCUSSION

More than 50% of cancer patients are affected by CC, characterized by a weight loss of at least 5% in 3 to 6 months or a body mass index (BMI) of <20.0 $kg.m^{-2}$. [109,110] The presence of an inflammatory state is frequently related with this condition, which is partly responsible for the increased muscle protein breakdown that leads to the severe and involuntary skeletal muscle loss observed in these patients. [61,110] It is important to notice that to a poor prognosis it is directly linked the colossal loss of skeletal muscle mass and that it is why it is considered the main hallmark of CC. [109] In fact, nearly 80% of patients with cancer suffering cachexia will be dead within 1 year post-diagnosis. [61] Furthermore, this multifactorial syndrome is accompanied by a reduction in the tolerance and effectiveness of anti-cancer treatments, increasing morbidity and further impairing the quality of life and survival of these patients. [109,110] In turn, chemotherapy, which is widely used in cancer treatment, might *per se* induce muscle wasting, thus exacerbating cachexia. [111] Unfortunately, conventional nutritional support cannot completely reverse the syndrome, since weight stabilization does not avoid the continuing loss of skeletal muscle mass or correct the related abnormal metabolic profile. [109,110] So, to prevent the wasting process it is necessary the implementation of mechanism-based therapeutic agents, but the poor knowledge of the underlying molecular mechanisms constitutes a problem. [109] Thus, treatments directed to muscle or inflammatory pathways may possibly be effective in decreasing the devastating effects of cachexia. [110]

One natural compound that has been studied as a potential therapy for wasting conditions is DMAPT, because it is capable of inhibiting the NF-κB pathway and presents anti-inflammatory effects, which are mechanisms responsible for CC. [70,108,112] Since other drugs targeting inflammation and the NF-κB pathway has been ascertained to counteract or reduce the devastating effects of CC, DMAPT seems a promising therapeutic approach for muscle wasting. [113,114] With these ideas in mind, we investigated the effect on the skeletal muscle of the widely-used chemotherapeutic agent cisplatin in healthy and bladder cancer-induced animals, and simultaneously, studied the possible therapeutic effect of the natural compound DMAPT to counteract the muscle wasting process.

In healthy animals, the acute administration of cisplatin (10 $mg.kg^{-1}$ at day zero) did not cause a reduction on the body weight nor gastrocnemius muscle weight (Table 1). These results suggest that an acute administration of cisplatin cannot induce body wasting, contrarily to the decrease on the body and muscle weights reported on healthy animals administered with 1 or 3 $mg.kg^{-1}$ of the drug daily for three or four days, respectively. [10,13] Furthermore, urothelial carcinoma did not induce *per se* muscle wasting (Table 2). However, when cisplatin was administered 4.0 $mg.kg^{-1}$ weekly for 6 weeks to tumour-bearing animals

the wasting syndrome occurred (Table 2). Thus, we can hypothesize that: i) chronic rather than acute cisplatin exposure seems to be necessary for inducing muscle wasting; ii) cancer and cisplatin possible have synergetic effects that, in overall, may cause a wasting process, visible on body and gastrocnemius muscle weights. Indeed, results of a study conducted by Rafael and collaborators [11] suggested the activation of mutual signalling pathways by both cancer and chemotherapy, leading to muscle wasting. Interestingly, only IL-6 appears to be involved in the cisplatin-induced wasting process in the context of cancer, since no differences were observed in the levels of the others inflammatory mediators studied (Figure 10). However, other cytokines, such as TNF-α and IL-1β, may be involved in the development of the condition. [54,115,116] Since in study 1 the levels of IL-6 in serum did not suffer any alteration with cisplatin administration (Figure 5), it can be suggested that this cytokine might have origin in tumour cells or in the immune response associated. Indeed, the primary sources of IL-6 are the tumour cells and immune cells, such as tumour-associated macrophages (TAM). [117] Interestingly, cisplatin caused a decrease in the spleen weight on urothelial-carcinoma-bearing animals (Table 2). Indeed, one study demonstrated that cisplatin may have adverse effects on the spleen, damaging its architecture, and therefore, decreasing its weight. [118] However, in healthy animals, did not occurred an increase in the spleen weight after cisplatin administration (Table 1) and any alteration in the serum levels of the inflammatory mediators studied (Figure 5) was observed, which is indicative of an absence of an inflammatory state, supporting the idea that no cachectic syndrome was developed. Furthermore, the absence of an enhanced proteolytic activity of the muscle-specific E3 ligases MuRF1 and MAFbx/atrogin-1 on the muscle (Figure 8) validates, at least, that acute administration of cisplatin, in healthy animals, does not induce a wasting effect. As suggested by Cloé and collaborators [119], the muscle mitochondria may be the place of energy wasting (diminution in ATP production efficiency) in cancer cachexia. So, the levels of ATP synthase, on whole gastrocnemius muscle, were evaluated and no differences were observed (Figure 6), which corroborates the nonexistence of a cachexia syndrome induced by a single dose of cisplatin in healthy animals. Cisplatin had an efficient chemotherapeutic function, since when only cisplatin was administered to BBN-animals, their body weight decreased comparatively with the BBN littermates (Table 2), possibly as a result of the diminution of the tumour size. This assumption can be corroborated with the decreased percentage of invasive and muscle invasive lesions in the cisplatin group (Table 3). In future work it will be important to analyse the cancer and cancer plus cisplatin-induced gastrocnemius muscle remodelling in order to view its anabolic/catabolic balance and to complete and confirm, or not, these results.

The natural compound DMAPT is currently target of several studies, because of its anti-inflammatory effect through the inhibition of NF-κB signalling. [120] Indeed, the increased gastrocnemius-to-body weight ratio observed in mice treated with cisplatin plus DMAPT, suggestive of hypertrophy (Table 1), might be due to the anti-inflammatory effect of this compound. These facts are corroborated by the levels of the acute-phase protein CRP (Figure 4), which may reflect the pro-inflammatory cytokine activity. [121] This molecule is a biomarker of inflammatory states and in the cisplatin plus DMAPT group its levels are decreased, which supports a decrease of inflammation. [122] It is also known that an increased expression of MMPs is presented in several inflammatory states, and so, the decrease of its levels on the muscle of cisplatin plus DMAPT animals comparatively to the cisplatin littermates (Figure 8) supports the previous. [123] Data concerning kidney weight (Table 1) might also support this hypothesis. An increase of kidney weight was observed on cisplatin-treated animals, which is indicative of cisplatin-induced renal damage. Indeed, nephrotoxicity is the principal dose-limiting toxicity of cisplatin, where occurs an accumulation of metabolites that cause direct inflammation. [124] Furthermore, Dilek and collaborators [125] observed edema in the kidneys of rats treated with cisplatin. Knowing that DMAPT can act as an anti-inflammatory agent, we can speculate that the reduction on the body weight is derived from a reduction of the inflammatory state and of the edema, leading to a body weight close to the control ones. [126] Regarding the spleen, cisplatin plus DMAPT decreased its weight (Table 1) that is consistent with a decrease in the splenic inflammatory responses. [127] In fact, cisplatin increases the splenic tissue expression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β and DMAPT in combination with the chemotherapeutic agent gemcitabine is capable of reducing the levels of these cytokines. [97,118] However, it seems that IL-6 does not play a fundamental role to the hypertrophy observed in the muscle of the animals treated with the combination of cisplatin and DMAPT, since the levels of this cytokine are similar between all the three groups.

Curiously, our results suggest that the combination of cisplatin plus DMAPT may induce the formation of p50 homodimers in the gastrocnemius muscle through the overexpression of TWEAK (Figure 7) and does not have an effect on the p65 subunit or IκBα. Indeed, TWEAK can activate the NF-κB pathway. [128] Comparing these results with other studies it was expected that wasting occurred, but interestingly, and as said before, the animals of this group seem to have developed hypertrophy, which should be confirmed by morphometric analysis of muscle sections. [13,129] Several cellular processes are regulated by the ubiquitously expressed factor NF-κB, including immune response, cellular survival, proliferation and differentiation. [130] Furthermore, it can be activated by numerous

extracellular signals, which emphasizes the complexity of its regulation and function. In addition, skeletal muscle development is a multistep process in which every stage is both positive and negatively regulated by mechanisms triggered by environmental stimulus, adding even more complexity to the skeletal muscle remodelling process mediated by NF-κB. [131] The majority of the studies regarding skeletal muscle injury refers that inflammation is linked to atrophy. [132] Nevertheless, this fact is not that straightforward, because of the closely relation between the regeneration of the skeletal myotubes and the inflammatory process. After an injury in the skeletal muscle, several types of cells, including macrophages, neutrophils and monocytes infiltrate into the damaged area. Simultaneously, satellite cells differentiate into transient-amplifying myoblasts that proliferate, fuse with one another and regenerate skeletal myotubes. Therefore, it is reasonable to presume that some mediators expressed during the inflammatory process may have an impact on skeletal muscle regeneration. Unfortunately, the exact mechanisms are unknown. [132] In this study, it seems that cisplatin plus DMAPT reduced the inflammation, and so, other pro-inflammatory cytokines besides the ones studied may be involved in this association between NF-κB, inflammation and hypertrophy. Another hypothesis is the association of the NF-κB pathway with the p38 MAPK one, since this cross-talk has been related with the control of the myogenic progression. [131] On one hand, the p38 MAPK pathway positively regulates the myogenic differentiation. On the other hand, the role of NF-κB is controversial, since both positive and negative effects have been stated. According to several studies, the NF-κB pathway is activated in proliferating and differentiating myoblasts and p38 activation induces NF-κB transcriptional activity. [131,133,134] It is also suggested by Lee and collaborators [135] that Akt, which is activated in response to some cytokines and has growth-promoting properties, may activate the NF-κB pathway through IKK and p38, stimulating p65 transactivation. [136] Therefore, in future studies it would be important to analyse these pathways and their relationship in the context of cancer- and cisplatin-induced muscle wasting.

Another positive effect of NF-κB on skeletal muscle is that during myoblast differentiation, IGF-2 can induce the NF-κB DNA-binding activity, possibly through a mechanism that involves IκBα phosphorylation. [137] There are also some studies regarding the occurrence of hypertrophy in the cardiac muscle. For example, upregulation of beclin 1 can induce cardiac hypertrophy and it is known that NF-κB positively regulates its expression. [138,139] Importantly, parthenolide, which is the sesquiterpene lactone from which DMAPT is generated, can increase the levels of beclin 1. [140] Another molecule that may be involved is the granulocyte colony-stimulating factor (G-CSF), since it participates

in cell differentiation, proliferation and survival and its expression can be induced by NF-κB activation. [132,141] This cytokine seems to have a critical role in skeletal myocyte regeneration, thus showing the importance of inflammation-mediated induction of muscle regeneration. [132] Besides all these studies, evidences of skeletal muscle hypertrophy induced by NF-κB activation are poor and in the context of cisplatin and/or DMAPT the studies are even lesser. So, we can only speculate that the combination of cisplatin and DMAPT, in healthy animals, possibly activates genes related with the differentiation process of myoblasts, leading to hypertrophy.

Since cisplatin in combination with DMAPT caused wasting on gastrocnemius of BBN animals (Table 2) suggests that DMAPT does not have protective effects on muscle when administered with cisplatin in cancer context. Nevertheless, it seems that DMAPT enhances the antitumoural activity of cisplatin, since no animals evidenced invasive and muscle invasive lesions (Table 3). Indeed, DMAPT showed anti-proliferative activity in tobacco-associated tumours. [142] Furthermore, it was ascertained in a study that DMAPT has cytotoxic effects towards breast cancer stem-like cells. [143] The natural compound DMAPT seems to be effective in reducing the inflammatory state induced by the tumour when combined with cisplatin, which can be observed by the spleen weight and the spleen-to-body weight ratio (Table 2).

VI. CONCLUSION AND FUTURE PERSPECTIVES

Overall, an acute administration of cisplatin, in healthy animals, appears to be incapable of induce a cachectic syndrome. Nevertheless, 15 days of DMAPT administration seems to induce muscle hypertrophy through the modulation of the inflammatory process and the TWEAK/NF-κB axis. However, when cancer is involved, cisplatin induces muscle wasting and DMAPT looks unable of reverting or ameliorating this process, despite its ability to reduce the inflammation, which in turn has an important role in the development of cachexia.

The mechanisms behind the muscle wasting process induced by chemotherapy are still to be understood, and unfortunately, few studies have been addressed to this specific problem. So, investigations devoted to the comprehension of how different chemotherapeutic agents (considering doses and mode of administration) may influence the loss of skeletal muscle mass are needed. Furthermore and in concordance with the results obtained in the present dissertation, it is important to ascertain the specific relation between the pathways activated by cancer itself and the ones induced by the anti-cancer drugs, and then the combination of both.

VII. BIBLIOGRAPHY

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